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**【Name of Document】** Abstract 1

**【General Power of Attorney Number】** 0216136

[Document Name] Claims

[Claim 1]

An antibody comprising a single-chain polypeptide having binding activity against TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions.

[Claim 2]

The antibody of claim 1, wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide.

[Claim 3]

The antibody of claim 1 or 2, wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers.

[Claim 4]

The antibody of claim 3, wherein the linkers comprise 15 amino acids.

[Claim 5]

A chimeric antibody that binds to Mpl.

[Claim 6]

The antibody of claim 5, which is a humanized antibody.

[Claim 7]

The antibody of claim 5 or 6, which is a minibody.

[Claim 8]

An antibody that binds to soluble Mpl.

[Claim 9]

An antibody that binds to human Mpl and monkey Mpl.

[Claim 10]

An antibody having agonistic activity against human Mpl and monkey Mpl.

[Claim 11]

An antibody whose binding activity to soluble Mpl is  $KD = 10^{-6}$  M or lower.

[Claim 12]

An antibody whose binding activity to soluble Mpl is  $KD = 10^{-7}$  M or lower.

[Claim 13]

An antibody whose binding activity to soluble Mpl is  $KD = 10^{-8}$  M or lower.

[Claim 14]

An antibody whose TPO agonistic activity is  $EC50 = 100$  nM or lower.

## [Claim 15]

An antibody whose TPO agonistic activity is  $EC_{50} = 30$  nM or lower.

## [Claim 16]

An antibody whose TPO agonistic activity is  $EC_{50} = 10$  nM or lower.

## 5 [Claim 17]

An antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

- 10 [1] SEQ ID NOs: 3, 4, and 5
- [2] SEQ ID NOs: 6, 7, and 8
- [3] SEQ ID NOs: 9, 10, and 11
- [4] SEQ ID NOs: 12, 13, and 14
- [5] SEQ ID NOs: 15, 16, and 17
- [6] SEQ ID NOs: 18, 19, and 20
- 15 [7] SEQ ID NOs: 21, 22, and 23
- [8] SEQ ID NOs: 24, 25, and 26
- [9] SEQ ID NOs: 27, 28, and 29
- [10] SEQ ID NOs: 30, 31, and 32
- [11] SEQ ID NOs: 33, 34, and 35
- 20 [12] SEQ ID NOs: 36, 37, and 38
- [13] SEQ ID NOs: 39, 40, and 41
- [14] SEQ ID NOs: 42, 43, and 44
- [15] SEQ ID NOs: 45, 46, and 47
- [16] SEQ ID NOs: 48, 49, and 50
- 25 [17] SEQ ID NOs: 51, 52, and 53
- [18] SEQ ID NOs: 54, 55, and 56
- [19] SEQ ID NOs: 57, 58, and 59
- [20] SEQ ID NOs: 231, 233, and 235
- [21] SEQ ID NOs: 266, 268, and 270
- 30 [22] SEQ ID NOs: 280, 282, and 284.

## [Claim 18]

An antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

- 35 [1] SEQ ID NOs: 60, 61, and 62
- [2] SEQ ID NOs: 63, 64, and 65



- [3] SEQ ID NOs: 66, 67, and 68  
[4] SEQ ID NOs: 69, 70, and 71  
[5] SEQ ID NOs: 72, 73, and 74  
[6] SEQ ID NOs: 75, 76, and 77  
5 [7] SEQ ID NOs: 78, 79, and 80  
[8] SEQ ID NOs: 81, 82, and 83  
[9] SEQ ID NOs: 84, 85, and 86  
[10] SEQ ID NOs: 87, 88, and 89  
[11] SEQ ID NOs: 90, 91, and 92  
10 [12] SEQ ID NOs: 93, 94, and 95  
[13] SEQ ID NOs: 96, 97, and 98  
[14] SEQ ID NOs: 99, 100, and 101  
[15] SEQ ID NOs: 102, 103, and 104  
[16] SEQ ID NOs: 105, 106, and 107  
15 [17] SEQ ID NOs: 108, 109, and 110  
[18] SEQ ID NOs: 111, 112, and 113  
[19] SEQ ID NOs: 114, 115, and 116  
[20] SEQ ID NOs: 240, 242, and 244  
[21] SEQ ID NOs: 273, 275, and 277.

20 [Claim 19]

An antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

- [1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that  
25 comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 60, 61, and 62;  
[2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID  
30 NOs: 63, 64, and 65;  
[3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 66, 67, and 68;  
35 [4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 12, 13, and 14, and a light chain variable region that

comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 69, 70, and 71;

5 [5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 72, 73, and 74;

10 [6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 75, 76, and 77;

[7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

15 [8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 81, 82, and 83;

20 [9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

25 [10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 87, 88, and 89;

30 [11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 90, 91, and 92;

[12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

35 [13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable

region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

[14] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable  
5 region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 99, 100, and 101;

[15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable  
10 region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

[16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable  
region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 105, 106, and 107;

15 [17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable  
region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110,

[18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising  
20 the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable  
region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

[19] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising  
25 the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable  
region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 114, 115, and 116;

[20] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising  
the amino acid sequences consisting of SEQ ID NOs: 231, 233, and 235, and a light chain  
variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences  
30 consisting of SEQ ID NOs: 240, 242, and 244;

[21] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising  
the amino acid sequences consisting of SEQ ID NOs: 266, 268, and 270, and a light chain  
variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences  
consisting of SEQ ID NOs: 273, 275, and 277;

35 [22] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising  
the amino acid sequences consisting of SEQ ID NOs: 280, 282, and 284, and a light chain

variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 273, 275, and 277.

[Claim 20]

An antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118.

[Claim 21]

An antibody that comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120.

[Claim 22]

An antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120.

[Claim 23]

An antibody comprising the amino acid sequence of SEQ ID NO: 122 or 264.

[Claim 24]

An antibody that comprises a heavy chain variable region, wherein said heavy chain variable region comprises FR1, FR2, FR3, and FR4 consisting of amino acid sequences of any one of:

[1] SEQ ID NOs: 230, 232, 234, and 236

[2] SEQ ID NOs: 265, 267, 269, and 271

[3] SEQ ID NOs: 279, 281, 283, and 285.

[Claim 25]

An antibody comprising a light chain variable region, wherein said light chain variable region comprises FR1, FR2, FR3, and FR4 consisting of amino acid sequences of any one of:

[1] SEQ ID NOs: 239, 241, 243, and 245

[2] SEQ ID NOs: 272, 274, 276, and 278.

[Claim 26]

An antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 230, 232, 234, and 236, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 239, 241, 243, and 245;

[2] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 265, 267, 269, and 271, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences

consisting of SEQ ID NOs: 272, 274, 276, and 278;

[3] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 279, 281, 283, and 285, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 272, 274, 276, and 278.

[Claim 27]

An antibody that comprises a heavy chain variable region, wherein said heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 229, 256, or 262.

[Claim 28]

An antibody that comprises a light chain variable region, wherein said light chain variable region comprises the amino acid sequence of SEQ ID NO: 238 or 258.

[Claim 29]

An antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 229, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 238;

[2] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 256, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

[3] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 262, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258.

[Claim 30]

An antibody that comprises the amino acid sequence of SEQ ID NO: 2, 254, or 260.

[Claim 31]

An antibody having an activity equivalent to that of an antibody of any one of claims 17 to 30, wherein said antibody comprises the amino acid sequence set forth in any one of claims 17 to 30, in which one or more amino acids have been substituted, deleted, added and/or inserted.

[Claim 32]

An antibody that recognizes an epitope recognized by an antibody of any one of claims 17 to 31.

[Claim 33]

An antibody that recognizes the region of amino acids 26 to 274 of human Mpl.

[Claim 34]

An antibody of any one of claims 1 to 33, which has TPO agonistic activity.

[Claim 35]

A polynucleotide encoding an antibody of any one of claims 1 to 34.

[Claim 36]



A polynucleotide hybridizing to the polynucleotide of claim 35 under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of claims 1 to 34.

[Claim 37]

5           A vector comprising the polynucleotide of claim 35 or 36.

[Claim 38]

A host cell that carries the polynucleotide of claim 35 or 36, or the vector of claim 37.

[Claim 39]

A pharmaceutical composition comprising an antibody of any one of claims 1 to 34.

[Document Name] Specification

[Title of the Invention] ANTI-MPL ANTIBODIES

[Technical Field]

5 [0001]

The present invention relates to anti-Mpl antibodies.

[Background of the Invention]

[0002]

10 Thrombopoietin (TPO) is a factor that enhances the differentiation and maturation of megakaryocytes (platelet precursor cells) from hemopoietic stem cells into platelets. TPO also functions as a cytokine with an important role in the regulation of platelet number. TPO is converted into its active form through the cleavage of a TPO precursor comprising 353 amino acids.

[0003]

15 Mpl is a TPO receptor, and human Mpl molecules are known to exist in two forms comprising 572 and 635 amino acids. The human Mpl gene sequence has already been analyzed (see Non-patent Document 1 and GenBank accession No. NM\_005373).

[0004]

20 Most cytokine receptors dimerize upon ligand binding, and transduce signals into cells. It has been reported that TPO similarly binds to its own specific receptor MPL, which leads to dimerization of the receptor, thereby transducing signals into cells and exerting physiological effects (see Non-patent Document 2).

[0005]

25 Antibodies exhibiting agonistic activity have been reported among those antibodies that bind to receptors having the above features.

For example, an antibody against the erythropoietin (EPO) receptor has been reported to substitute for erythropoietin function. The monovalent form (Fab) of the antibody is capable of binding to the EPO receptor but is unable to transduce signals. Thus, dimerization of the erythropoietin receptor *via* bivalent binding is assumed to be essential for signal transduction  
30 (see Non-patent Document 3).

[0006]

Antibodies that bind to Mpl and exhibit TPO agonistic activity have also been reported (see Non-patent Documents 4 and 5). This suggests that receptor dimerization is induced upon binding of a bivalent antibody with regards to MPL as well.

35 [0007]

Meanwhile, a single-chain antibody (scFv) has been reported to exhibit TPO agonistic

activity (see Patent Document 1). However, it has been revealed that, the underlying mechanism of scFv exhibiting TPO agonistic activity is that a part of scFv dimerizes (diabody) and this diabody becomes the actual active unit (see Patent Documents 2 to 4).

[0008]

[Patent Document 1] US Patent No. 6342220

[Patent Document 2] WO 01/79494

[Patent Document 3] WO 02/33072

[Patent Document 4] WO 02/33073

[Non-patent Document 1] Palacios *et al.*, 1985, Cell, 41, 727-734

[Non-patent Document 2] Souyri *et al.*, 1990, Cell, Vol.63, 1137-1147

[Non-patent Document 3] Elliott, S. *et al.*, 1996, J. Biol. Chem., 271(40), 24691-24697

[Non-patent Document 4] Abe *et al.*, 1998, Immunol. Lett., 61, 73-78

[Non-patent Document 5] Bijia Deng *et al.*, 1998, Blood, 92, 1981-1988

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

[0009]

The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide novel anti-Mpl antibodies having TPO agonistic activity.

[Means for Solving the Problems]

[0010]

The present inventors performed exhaustive research to solve the above objective. The present inventors prepared and purified anti-human Mpl antibody VB22B, and established a single-chain antibody expression system using genetic engineering techniques. Specifically, the variable region of anti-human Mpl antibody was first cloned, and a diabody expression vector pCXND3-VB22B db for the anti-human Mpl antibody was prepared. This pCXND3-VB22B db vector was then used to generate an expression vector pCXND3-VB22B sc(Fv)<sub>2</sub> for anti-human Mpl antibody sc(Fv)<sub>2</sub>. Anti-human Mpl sc(Fv)<sub>2</sub> was expressed in CHO-DG44 cells using the expression vector pCXND3-VB22B sc(Fv)<sub>2</sub>, and then purified from the culture supernatant. In control experiments, VB22B diabody was transiently expressed in COS7 cells using the above pCXND3-VB22B db vector, and then purified from the culture supernatant.

[0011]

In addition, VB22B diabody and VB22B sc(Fv)<sub>2</sub> were evaluated for their TPO-like agonistic activities. The results showed that VB22B diabody and VB22B sc(Fv)<sub>2</sub> exhibit higher agonistic activities compared to VB22B IgG, and thus activities equivalent to or higher than that of the natural ligand, human TPO.

[0012]



Furthermore, the present inventors succeeded in preparing three types of humanized VB22B sc(Fv)<sub>2</sub>. The TPO-like agonistic activity was also proven to be unaltered by humanization.

[0013]

More specifically, the present invention relates to:

(1) an antibody comprising a single-chain polypeptide having binding activity against TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions;

(2) the antibody of (1), wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide;

(3) the antibody of (1) or (2), wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers;

(4) the antibody of (3), wherein the linkers comprise 15 amino acids;

(5) a chimeric antibody that binds to Mpl;

(6) the antibody of (5), which is a humanized antibody;

(7) the antibody of (5) or (6), which is a minibody;

(8) an antibody that binds to soluble Mpl;

(9) an antibody that binds to human Mpl and monkey Mpl;

(10) an antibody having agonistic activity against human Mpl and monkey Mpl;

(11) an antibody whose binding activity to soluble Mpl is  $KD = 10^{-6}$  M or lower;

(12) an antibody whose binding activity to soluble Mpl is  $KD = 10^{-7}$  M or lower;

(13) an antibody whose binding activity to soluble Mpl is  $KD = 10^{-8}$  M or lower;

(14) an antibody whose TPO agonistic activity is  $EC50 = 100$  nM or lower;

(15) an antibody whose TPO agonistic activity is  $EC50 = 30$  nM or lower;

(16) an antibody whose TPO agonistic activity is  $EC50 = 10$  nM or lower;

(17) an antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 3, 4, and 5

[2] SEQ ID NOs: 6, 7, and 8

[3] SEQ ID NOs: 9, 10, and 11

[4] SEQ ID NOs: 12, 13, and 14

[5] SEQ ID NOs: 15, 16, and 17

[6] SEQ ID NOs: 18, 19, and 20

[7] SEQ ID NOs: 21, 22, and 23

[8] SEQ ID NOs: 24, 25, and 26

[9] SEQ ID NOs: 27, 28, and 29

[10] SEQ ID NOs: 30, 31, and 32

5 [11] SEQ ID NOs: 33, 34, and 35

[12] SEQ ID NOs: 36, 37, and 38

[13] SEQ ID NOs: 39, 40, and 41

[14] SEQ ID NOs: 42, 43, and 44

[15] SEQ ID NOs: 45, 46, and 47

10 [16] SEQ ID NOs: 48, 49, and 50

[17] SEQ ID NOs: 51, 52, and 53

[18] SEQ ID NOs: 54, 55, and 56

[19] SEQ ID NOs: 57, 58, and 59

[20] SEQ ID NOs: 231, 233, and 235

15 [21] SEQ ID NOs: 266, 268, and 270

[22] SEQ ID NOs: 280, 282, and 284;

(18) an antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

20 [1] SEQ ID NOs: 60, 61, and 62

[2] SEQ ID NOs: 63, 64, and 65

[3] SEQ ID NOs: 66, 67, and 68

[4] SEQ ID NOs: 69, 70, and 71

[5] SEQ ID NOs: 72, 73, and 74

25 [6] SEQ ID NOs: 75, 76, and 77

[7] SEQ ID NOs: 78, 79, and 80

[8] SEQ ID NOs: 81, 82, and 83

[9] SEQ ID NOs: 84, 85, and 86

[10] SEQ ID NOs: 87, 88, and 89

30 [11] SEQ ID NOs: 90, 91, and 92

[12] SEQ ID NOs: 93, 94, and 95

[13] SEQ ID NOs: 96, 97, and 98

[14] SEQ ID NOs: 99, 100, and 101

[15] SEQ ID NOs: 102, 103, and 104

35 [16] SEQ ID NOs: 105, 106, and 107

[17] SEQ ID NOs: 108, 109, and 110

[18] SEQ ID NOs: 111, 112, and 113

[19] SEQ ID NOs: 114, 115, and 116

[20] SEQ ID NOs: 240, 242, and 244

[21] SEQ ID NOs: 273, 275, and 277;

5 (19) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting  
10 of SEQ ID NOs: 60, 61, and 62;

[2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

15 [3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 66, 67, and 68;

[4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising  
20 the amino acid sequences consisting of SEQ ID NOs: 12, 13, and 14, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 69, 70, and 71;

[5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable  
25 region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 72, 73, and 74;

[6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting  
30 of SEQ ID NOs: 75, 76, and 77;

[7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

35 [8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable

region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 81, 82, and 83;

5 [9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

10 [10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 87, 88, and 89;

[11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 90, 91, and 92;

15 [12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

20 [13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

25 [14] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 99, 100, and 101;

30 [15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

[16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 105, 106, and 107;

35 [17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable

region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110;

[18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable  
5 region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

[19] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable  
10 region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 114, 115, and 116;

[20] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 231, 233, and 235, and a light chain  
variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 240, 242, and 244;

[21] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 266, 268, and 270, and a light chain  
15 variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 273, 275, and 277;

[22] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 280, 282, and 284, and a light chain  
20 variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 273, 275, and 277;

(20) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118;

[21] an antibody that comprises a light chain variable region comprising the amino acid  
25 sequence of SEQ ID NO: 120;

(22) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid  
sequence of SEQ ID NO: 120;

[23] an antibody comprising the amino acid sequence of SEQ ID NO: 122 or 264;

[24] an antibody that comprises a heavy chain variable region, wherein said heavy chain  
variable region comprises FR1, FR2, FR3, and FR4 consisting of amino acid sequences of any  
one of:

[1] SEQ ID NOs: 230, 232, 234, and 236

[2] SEQ ID NOs: 265, 267, 269, and 271

[3] SEQ ID NOs: 279, 281, 283, and 285.



(25) an antibody comprising a light chain variable region, wherein said light chain variable region comprises FR1, FR2, FR3, and FR4 consisting of amino acid sequences of any one of:

[1] SEQ ID NOs: 239, 241, 243, and 245

[2] SEQ ID NOs: 272, 274, 276, and 278;

(26) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 230, 232, 234, and 236, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 239, 241, 243, and 245;

[2] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 265, 267, 269, and 271, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 272, 274, 276, and 278;

[3] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 279, 281, 283, and 285, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 272, 274, 276, and 278;

(27) an antibody that comprises a heavy chain variable region, wherein said heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 229, 256, or 262;

(28) an antibody that comprises a light chain variable region, wherein said light chain variable region comprises the amino acid sequence of SEQ ID NO: 238 or 258;

(29) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 229, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 238;

[2] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 256, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

[3] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 262, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

(30) an antibody that comprises the amino acid sequence of SEQ ID NO: 2, 254, or 260;

(31) an antibody having an activity equivalent to that of an antibody of any one of (17) to (30), wherein said antibody comprises the amino acid sequence set forth in any one of (17) to (30), in which one or more amino acids have been substituted, deleted, added and/or inserted;

(32) an antibody that recognizes an epitope recognized by an antibody of any one of

(17) to (31);

(33) an antibody that recognizes the region of amino acids 26 to 274 of human Mpl;

(34) an antibody of any one of (1) to (33), which has TPO agonistic activity;

(35) a polynucleotide encoding an antibody of any one of (1) to (34);

5 (36) a polynucleotide hybridizing to the polynucleotide of (35) under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of (1) to (34);

(37) a vector comprising the polynucleotide of (35) or (36);

(38) a host cell that carries the polynucleotide of (35) or (36), or the vector of (37); and

10 (39) a pharmaceutical composition comprising an antibody of any one of (1) to (34).

[Effects of the Invention]

[0014]

Recombinant human TPOs have been tested as therapeutic agents for chemotherapy-induced thrombocytopenia in various clinical trials. In these trials, a major  
15 problem that was reported was the production of anti-TPO antibody in TPO-treated patients (Junzhi Li, *et al.*, Blood (2001) 98: 3241-324; Saroj Vandhan-Raj, *et al.*, Ann. Intern. Med. (2000) 132: 364-368). Specifically, production of neutralizing antibodies that inhibit endogenous TPO activity, and the resulting onset of thrombocytopenia, were reported. Administration of agonistic minibodies (low molecular weight antibodies) against anti-TPO  
20 receptor of the present invention does not induce the production of antibodies against endogenous TPO. Furthermore, since antibody miniaturization results in increased specific activity and short half-life in blood, the effective concentration in blood can be easily regulated, presenting a further clinical advantage. Accordingly, the present antibodies are expected to be therapeutic agents for thrombocytopenia which are more effective than naturally-occurring TPO  
25 or its agonistic antibodies. Furthermore, since minibodies are not glycosylated, expression systems for expressing the recombinant proteins are not limited, and minibodies can be produced in any expression system such as cell lines from mammals, yeast, insect cells, and *E. coli*. Since binding specificity against mutant TPO receptor is different from that of TPO, minibodies are expected to bind specific mutants and show agonistic activity against mutated TPO receptor  
30 detected in the CAMT patients, who genetically have mutated TPO receptor and develop thrombocytopenia.

[Best Mode for Carrying Out the Invention]

[0015]

The present invention provides antibodies that bind to the TPO receptor (Mpl).

35 [0016]

The antibodies of the present invention comprise all types of antibodies, including

antibodies with modified amino acid sequences, such as minibodies, humanized antibodies, and chimeric antibodies; antibodies that have been modified by binding with other molecules (for example, polymers such as polyethylene glycol); and antibodies whose sugar chains have been modified.

5 [0017]

Mpl of the present invention may be a mutant receptor. A mutant receptor of the present invention is usually a receptor that exists at a frequency lower than 50%, preferably lower than 20%, more preferably lower than 10%, and even more preferably lower than 1%.

The frequency is generally calculated using randomly selected subjects. However, the

10 frequency may vary depending on the country, area, sex, and such. Therefore, the frequency may also be calculated, for example, within a defined country or area, such as Japan, the United States, and Europe, or calculated for one sex. When there are two or more mutations in a receptor, the frequency may be calculated for multiple mutation sites or for any one of the mutation sites. Mutant receptors are preferably evaluated by a frequency as described above.

15 However, mutant receptors can also be evaluated, for example, by their signal transducing ability and such. Specifically, for example, when two different receptors are present, the one with stronger transducing signals upon natural ligand-binding maybe be used as a non-mutant type receptor, and the one with weaker transducing signals as a mutant receptor.

[0018]

20 In one embodiment, the mutant receptors of the present invention comprise receptors that are associated with disease onset. The phrase "mutant receptors associated with disease onset" means that the loss of reactivity to a natural ligand becomes part of the reason that triggers disease onset. In the present invention, the mutant receptor may be a contributing factor, but not necessarily the sole factor triggering disease onset. Many reports have been

25 previously published that describe the association of mutant receptors with disease onset. In addition to those that have been reported, associations of mutant receptors and disease onset can also be identified by statistical analysis methods (for example, correlation analyses).

Correlation analyses, also called "case control studies", are well known to those skilled in the art (for example, Nishimura, Y., 1991, "Statistical analysis of polymorphisms", Saishin Igaku,

30 46:909-923; Oka, A. *et al.*, Hum. Mol. Genetics (1990) 8: 2165-2170; Ota, M. *et al.*, Am. J. Hum. Genet. (1999) 64: 1406-1410; Ozawa, A. *et al.*, Tissue Antigens (1999) 53: 263-268). For

example, the correlation between a mutant receptor and a disease can be studied by computing the frequency of the mutant receptor in patients and healthy subjects, and testing whether the patient population has a higher mutant receptor frequency. Typically, differences in frequency

35 are evaluated using the  $\chi$ -test.  $\chi$  is obtained by the equation  $\chi^2 = \sum (\text{observed value} - \text{expected value})^2 / \text{expected value}$ . A p value is obtained from the  $\chi^2$  value determined. Based on this p



value, it can be determined whether there is a correlation between the mutant receptor and the disease. For example, when  $p < 0.05$ , the mutant receptor is considered to correlate with the disease. Mutant thrombopoietin (TPO) receptors have already been reported (Matthias Ballmaier *et al.*, BLOOD (2001) 97 (1): 139; and others).

5 [0019]

It is preferable that the antibodies of the present invention have agonistic activity against Mpl.

In a preferred embodiment, the antibodies of the present invention comprise, for example, minibodies.

10 The minibodies comprise antibody fragments lacking portions of the whole antibody (for example, whole IgG). The minibodies are not particularly limited as long as they have binding activity to their antigens. The minibodies of the present invention have markedly higher activities compared to their corresponding whole antibodies. There are no particular limitations on the antibody fragments of the present invention as long as they are portions of the  
15 whole antibody, and preferably contain heavy chain variable regions (VH) and/or light chain variable regions (VL). The amino acid sequences of VH or VL may contain substitutions, deletions, additions and/or insertions. Furthermore, the antibody fragment may also lack portions of VH or/and VL, as long as it has binding ability to its antigen. In addition, the variable regions may be chimerized or humanized. Such antibody fragments include, for  
20 example, Fab, Fab', F(ab')<sub>2</sub>, and Fv. An example of a minibody includes Fab, Fab', F(ab')<sub>2</sub>, Fv, scFv (single-chain Fv), diabody, and sc(Fv)<sub>2</sub> (single-chain (Fv)<sub>2</sub>).

[0020]

Herein, an "Fv" fragment is the smallest antibody fragment and contains a complete antigen recognition site and a binding site. The "Fv" fragment is a dimer (VH-VL dimer) in  
25 which a single VH and a single VL are strongly linked by a non-covalent bond. The three complementarity-determining regions (CDRs) of each of the variable regions interact with each other to form an antigen-binding site on the surface of the VH-VL dimer. Six CDRs confer the antigen-binding site of an antibody. However, a single variable region (or a half of Fv containing only three CDRs specific to an antigen) alone is also capable of recognizing and  
30 binding an antigen although its affinity is lower than the affinity of the entire binding site.

[0021]

scFv contains the VH and VL regions of an antibody, and these regions exist on a single polypeptide chain. Generally, an Fv polypeptide further contains a polypeptide linker between VH and VL, and therefore an scFv can form a structure required for antigen binding. See,  
35 Pluckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113 (Rosenburg and Moore eds. (Springer Verlag, New York, pp.269-315, 1994) for the review of scFv. In the present invention,

linkers are not especially limited as long as they do not inhibit expression of antibody variable regions linked at both ends of the linkers.

[0022]

The term “diabody” refers to a bivalent antibody fragment constructed by gene fusion  
 5 (Holliger P *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90: 6444-6448; EP 404,097; WO 93/11161 and others). Diabodies are dimers comprising two polypeptide chains, where each polypeptide chain comprises a VL and a VH connected with a linker short enough to prevent interaction of these two domains, for example, a linker of about five residues. The VL and VH encoded on  
 10 the same polypeptide chain will form a dimer because the linker between them is too short to form a single-chain variable region fragment. As a result, the polypeptide chains form a dimer, and thus the diabody has two antigen binding sites.

[0023]

sc(Fv)<sub>2</sub> is a single-chain minibody produced by linking two units of VH and two units of VL with linkers and such (Hudson *et al.*, J Immunol. Methods (1999) 231:177-189). sc(Fv)<sub>2</sub>  
 15 exhibits a particularly high agonistic activity compared to the whole antibody and other minibodies. sc(Fv)<sub>2</sub> can be produced, for example, by linking two scFv molecules.

In a preferable antibody, the two VH units and two VL units are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide.

20 [0024]

The order of the two VH units and two VL units is not limited to the above arrangement, and they may be arranged in any order. Examples of the arrangements are listed below.

[VL]-linker-[VH]-linker-[VH]-linker-[VL]

[VH]-linker-[VL]-linker-[VL]-linker-[VH]

25 [VH]-linker-[VH]-linker-[VL]-linker-[VL]

[VL]-linker-[VL]-linker-[VH]-linker-[VH]

[VL]-linker-[VH]-linker-[VL]-linker-[VH]

[0025]

The linkers to be used for linking the variable regions of an antibody comprise arbitrary  
 30 peptide linkers that can be introduced by genetic engineering, synthetic linkers, and linkers disclosed in, for example, Holliger, P. *et al.*, Protein Engineering (1996) 9 (3): 299-305. Peptide linkers are preferred in the present invention. There are no limitations as to the length of the peptide linkers. The length can be selected accordingly by those skilled in the art depending on the purpose, and is typically 1-100 amino acids, preferably 3-50 amino acids, more  
 35 preferably 5-30 amino acids, and even more preferably 12-18 amino acids (for example, 15 amino acids).

[0026]

For example, such peptide linkers include:

Ser

Gly Ser

5 Gly Gly Ser

Ser Gly Gly

Gly Gly Gly Ser

Ser Gly Gly Gly

Gly Gly Gly Gly Ser

10 Ser Gly Gly Gly Gly

Gly Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly Gly

Gly Gly Gly Gly Gly Gly Ser

Ser Gly Gly Gly Gly Gly Gly

15 (Gly Gly Gly Gly Ser)<sub>n</sub>

(Ser Gly Gly Gly Gly)<sub>n</sub>

where n is an integer of 1 or larger. The lengths and sequences of peptide linkers can be selected accordingly by those skilled in the art depending on the purpose.

In an embodiment of the present invention, a particularly preferable sc(Fv)<sub>2</sub> includes,  
20 for example, the sc(Fv)<sub>2</sub> below.

[0027]

[VH]-peptide linker (15 amino acids)-[VL]-peptide linker (15 amino acids)-[VH]-peptide linker (15 amino acids)-[VL]

[0028]

25 Synthetic linkers (chemical crosslinking agents) include crosslinking agents routinely used to crosslink peptides, for example, N-hydroxy succinimide (NHS), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS<sup>3</sup>), dithiobis(succinimidyl propionate) (DSP), dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidoxycarbonyloxy)ethyl]  
30 sulfone (BSOCOES), and bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES). These crosslinking agents are commercially available.

[0029]

In general, three linkers are required to link four antibody variable regions together.  
35 The linkers to be used may be of the same type or different types. In the present invention, a preferable minibody is a diabody, even more preferably, an sc(Fv)<sub>2</sub>. Such a minibody can be

prepared by treating an antibody with an enzyme, for example, papain or pepsin, to generate antibody fragments, or by constructing DNAs encoding those antibody fragments and introducing them into expression vectors, followed by expression in an appropriate host cell (see, for example, Co, M. S. *et al.*, J. Immunol. (1994) 152: 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178: 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178: 497-515; Lamoyi, E., Methods Enzymol. (1986) 121: 652-663; Rousseaux, J. *et al.*, Methods Enzymol. (1986) 121: 663-669; Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9: 132-137).

[0030]

10 An antibody having exceedingly high agonistic activity can be prepared by reducing the molecular weight of a full-length antibody, particularly by converting it into an sc(Fv)<sub>2</sub>.

In a preferred embodiment, the antibodies of the present invention comprise modified antibodies, such as chimeric antibodies and humanized antibodies that bind to Mpl. These modified antibodies can be produced by known methods.

15 [0031]

Chimeric antibodies are antibodies prepared by combining sequences derived from different animal species, and include for example, antibodies comprising the heavy chain and light chain variable regions of a murine antibody, and the heavy chain and light chain constant regions of a human antibody. Chimeric antibodies can be prepared by known methods. For example, a DNA encoding the V region of an antibody is linked to a DNA encoding the C region of a human antibody, and the construct is inserted into an expression vector and introduced into a host to produce chimeric antibodies.

[0032]

25 Humanized antibodies are also referred to as “reshaped human antibodies”. Such a humanized antibody is obtained by transferring the complementarity-determining region (CDR) of an antibody derived from a non-human mammal, for example mouse, to the complementarity-determining region of a human antibody, and the general gene recombination procedure for this is also known (see European Patent Application No. 125023 and WO 96/02576).

30 [0033]

Specifically, a DNA sequence designed to link a murine antibody CDR to the framework region (FR) of a human antibody can be synthesized by PCR, using primers prepared from several oligonucleotides containing overlapping portions of both CDR and FR terminal regions (see methods described in WO 98/13388).

35 [0034]

The human antibody framework region to be linked by CDR is selected in order to form

a favorable antigen-binding site in the complementarity-determining region. Amino acids of the framework region in the antibody variable region may be substituted, as necessary, for the complementarity-determining region of the reshaped human antibody to form a suitable antigen-binding site (Sato, K. *et al.*, Cancer Res. (1993) 53: 851-856).

5 [0035]

The constant region of a human antibody is used as the constant region of a chimeric antibody or humanized antibody. For example, C $\gamma$ 1, C $\gamma$ 2, C $\gamma$ 3, and C $\gamma$ 4 can be used as the H chain, and C $\kappa$  and C $\lambda$  can be used as the L chain. The human antibody constant region may be modified to improve the antibody or the stability of the antibody production.

10 [0036]

Generally, chimeric antibodies comprise the variable region of an antibody from a non-human mammal and the constant region derived from a human antibody. On the other hand, humanized antibodies comprise the complementarity-determining region of an antibody from a non-human mammal, and the framework region and constant region derived from a human antibody.

15 [0037]

In addition, after a chimeric antibody or a humanized antibody is prepared, amino acids in the variable region (for example, FR) and the constant region may be replaced with other amino acids, and such.

20 The origin of the variable regions in chimeric antibodies or that of the CDRs in humanized antibodies is not particularly limited, and may be derived from any type of animal. For example, sequences of murine antibodies, rat antibodies, rabbit antibodies, camel antibodies may be used.

[0038]

25 In general, it is difficult to chimerize or humanize an antibody without losing the agonistic activity of the original antibody. Nevertheless, the present invention succeeded in preparing humanized antibodies having agonistic activity equivalent to that of the original murine antibody.

[0039]

30 A preferred humanized antibody of the present invention is an antibody comprising a heavy chain variable region that comprises the amino acid sequence of SEQ ID NO: 229 (humanized heavy chain sequence: hVB22B p-z VH), SEQ ID NO: 256 (humanized heavy chain sequence: hVB22B g-e VH), or SEQ ID NO: 262 (humanized heavy chain sequence: hVB22B e VH); or an antibody comprising a light chain variable region that comprises the amino acid sequence of SEQ ID NO: 238 (humanized light chain hVB22B p-z VL), or SEQ ID NO: 258 (humanized light chain hVB22B g-e VL or hVB22B e VL). In particular, a preferred antibody

35



is an antibody comprising a heavy chain variable region and a light chain variable region of any one of (1) to (3) indicated below:

(1) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 229, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 238;

5 (2) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 256, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

(3) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 262, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258.

[0040]

10 Such antibodies include, for example, antibodies comprising the amino acid sequence of SEQ ID NO: 2, 254, or 260 (humanized sc(Fv)<sub>2</sub> sequence (hVB22B p-z sc(Fv)<sub>2</sub>, hVB22B g-e sc(Fv)<sub>2</sub>, or hVB22B e sc(Fv)<sub>2</sub>).

[0041]

15 The nucleotide sequence of hVB22B p-z VH is shown in SEQ ID NO: 228; the nucleotide sequence of hVB22B g-e VH is shown in SEQ ID NO: 255; the nucleotide sequence of hVB22B e VH is shown in SEQ ID NO: 261; the nucleotide sequence of hVB22B p-z VL is shown in SEQ ID NO: 237; the nucleotide sequences of hVB22B g-e VL and hVB22B e VL are shown in SEQ ID NO: 257.

[0042]

20 In the amino acid sequence of SEQ ID NO: 229 (humanized heavy chain sequence: hVB22B p-z VH), SEQ ID NO: 256 (humanized heavy chain sequence: hVB22B g-e VH), or SEQ ID NO: 262 (humanized heavy chain sequence: hVB22B e VH), amino acids 31-35 correspond to CDR1; amino acids 50-66 correspond to CDR2; amino acids 99-107 correspond to CDR3; amino acids 1-30 correspond to FR1; amino acids 36-49 correspond to FR2; amino acids 67-98 correspond to FR3; and amino acids 108-118 correspond to FR4.

30 [0043]

In the amino acid sequence of SEQ ID NO: 238 (humanized light chain sequence: hVB22B p-z VL) or SEQ ID NO: 258 (humanized light chain sequence: hVB22B g-e VL or hVB22B e VL), amino acids 24-39 correspond to CDR1; amino acids 55-61 correspond to CDR2; amino acids 94-102 correspond to CDR3;

amino acids 1-23 correspond to FR1;  
amino acids 40-54 correspond to FR2;  
amino acids 62-93 correspond to FR3; and  
amino acids 103-112 correspond to FR4.

5 [0044]

In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B p-z VH sequence are shown below:

hVB22B p-z VH: FR1/SEQ ID NO: 230

hVB22B p-z VH: CDR1/SEQ ID NO: 231

10 hVB22B p-z VH: FR2/SEQ ID NO: 232

hVB22B p-z VH: CDR2/SEQ ID NO: 233

hVB22B p-z VH: FR3/SEQ ID NO: 234

hVB22B p-z VH: CDR3/SEQ ID NO: 235

hVB22B p-z VH: FR4/SEQ ID NO: 236.

15 [0045]

In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B p-z VL sequence are shown below:

hVB22B p-z VL: FR1/SEQ ID NO: 239

hVB22B p-z VL: CDR1/SEQ ID NO: 240

20 hVB22B p-z VL: FR2/SEQ ID NO: 241

hVB22B p-z VL: CDR2/SEQ ID NO: 242

hVB22B p-z VL: FR3/SEQ ID NO: 243

hVB22B p-z VL: CDR3/SEQ ID NO: 244

hVB22B p-z VL: FR4/SEQ ID NO: 245.

25 [0046]

In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B g-e VH sequence are shown below:

hVB22B g-e VH: FR1/SEQ ID NO: 265

hVB22B g-e VH: CDR1/SEQ ID NO: 266

30 hVB22B g-e VH: FR2/SEQ ID NO: 267

hVB22B g-e VH: CDR2/SEQ ID NO: 268

hVB22B g-e VH: FR3/SEQ ID NO: 269

hVB22B g-e VH: CDR3/SEQ ID NO: 270

hVB22B g-e VH: FR4/SEQ ID NO: 271.

35 [0047]

In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B g-e VL

sequence are shown below:

hVB22B g-e VL: FR1/SEQ ID NO: 272

hVB22B g-e VL: CDR1/SEQ ID NO: 273

hVB22B g-e VL: FR2/SEQ ID NO: 274

5 hVB22B g-e VL: CDR2/SEQ ID NO: 275

hVB22B g-e VL: FR3/SEQ ID NO: 276

hVB22B g-e VL: CDR3/SEQ ID NO: 277

hVB22B g-e VL: FR4/SEQ ID NO: 278.

[0048]

10 In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B e VH sequence are shown below:

hVB22B e VH: FR1/SEQ ID NO: 279

hVB22B e VH: CDR1/SEQ ID NO: 280

hVB22B e VH: FR2/SEQ ID NO: 281

15 hVB22B e VH: CDR2/SEQ ID NO: 282

hVB22B e VH: FR3/SEQ ID NO: 283

hVB22B e VH: CDR3/SEQ ID NO: 284

hVB22B e VH: FR4/SEQ ID NO: 285.

[0049]

20 In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B e VL sequence are shown below:

hVB22B e VL: FR1/SEQ ID NO: 272

hVB22B e VL: CDR1/SEQ ID NO: 273

hVB22B e VL: FR2/SEQ ID NO: 274

25 hVB22B e VL: CDR2/SEQ ID NO: 275

hVB22B e VL: FR3/SEQ ID NO: 276

hVB22B e VL: CDR3/SEQ ID NO: 277

hVB22B e VL: FR4/SEQ ID NO: 278.

[0050]

30 SEQ ID NOs of the CDRs and FRs in the hVB22B p-z VH sequence and hVB22B p-z VL sequence are shown in Figure 18.

[0051]

In other embodiments, preferred humanized antibodies of the present invention include:  
humanized antibodies comprising a heavy chain variable region which has FR1, 2, 3, and 4  
35 comprising amino acid sequences of any one of (1) to (3) indicated below:

(1) SEQ ID NOs: 230, 232, 234, and 236,



(2) SEQ ID NOs: 265, 267, 269, and 271,

(3) SEQ ID NOs: 279, 281, 283, and 285;

humanized antibodies comprising a light chain variable region which has FR1, 2, 3, and 4 comprising amino acid sequences of (1) or (2) listed below:

5 (1) SEQ ID NOs: 239, 241, 243, and 245,

(2) SEQ ID NOs: 272, 274, 276, and 278;

humanized antibodies comprising a heavy chain variable region which has CDR1, 2 and 3 comprising amino acid sequences of any one of (1) to (3) listed below:

(1) SEQ ID NOs: 231, 233, and 235,

10 (2) SEQ ID NOs: 266, 268, and 270,

(3) SEQ ID NOs: 280, 282, and 284; and

humanized antibodies comprising a light chain variable region which has CDR1, 2 and 3 comprising amino acid sequences of (1) or (2) listed below:

(1) SEQ ID NOs: 240, 242, and 244,

15 (2) SEQ ID NOs: 273, 275, and 277.

[0052]

In yet another preferred embodiments, preferred humanized antibodies of the present invention include:

humanized antibodies comprising heavy chain and light chain variable regions of any one of (1)  
20 to (3) indicated below.

(1) a heavy chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 230, 232, 234, and 236, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 239, 241, 243, and 245, respectively;

25 (2) a heavy chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 265, 267, 269, and 271, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively;

30 (3) a heavy chain variable region which comprises FR1, 2, 3 and 4 comprising the amino acid sequences of SEQ ID NOs: 279, 281, 283, and 285, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively; and

humanized antibodies comprising heavy chain and light chain variable regions of any one of (1) to (3) indicated below:

35 (1) a heavy chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 231, 233, and 235, respectively, and a light chain variable region

which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 240, 242, and 244, respectively;

(2) a heavy chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 266, 268, and 270, respectively, and a light chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 273, 275, and 277, respectively;

(3) a heavy chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 280, 282, and 284, respectively, and a light chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 273, 275, and 277, respectively.

[0053]

Chimeric antibodies and humanized antibodies exhibit lower antigenicity in the human body, and thus are expected to be useful when administered to humans for therapeutic purposes.

In one embodiment, the preferred antibodies of the present invention include antibodies that bind to soluble Mpl. The term "soluble Mpl" herein refers to Mpl molecules excluding those expressed on the cell membrane. A specific example of a soluble Mpl is an Mpl lacking the entire or a portion of the transmembrane domain. The transmembrane domain of human Mpl corresponds to amino acids 492 to 513 in SEQ ID NO: 123.

[0054]

An antibody that binds to soluble recombinant Mpl can be used in detailed epitope analysis and kinetic analysis of receptor-ligand binding, as well as for assessing the blood concentration and dynamic behavior of the antibody in *in vivo* tests.

In one embodiment, the preferred antibodies of the present invention include antibodies having binding activity against both human and monkey Mpl. Antibodies having agonistic activity to both human and monkey Mpl are expected to be highly useful since the dynamic behavior and *in vivo* effects of the antibody, which are generally difficult to determine in human body, can be examined with monkeys.

[0055]

The present invention also provides antibodies having agonistic activity to human Mpl and monkey Mpl. Such antibodies may also have binding activity or agonistic activity against Mpl from animals other than humans and monkeys (for example, mice).

[0056]

In addition, the antibodies of the present invention include antibodies with TPO agonistic activity (agonistic activity against Mpl) of  $EC_{50} = 100$  nM or lower, preferably  $EC_{50} = 30$  nM or lower, more preferably  $EC_{50} = 10$  nM or lower.

[0057]

The agonistic activity can be determined by methods known to those skilled in the art, for example, by the method described below.

The sequences for human Mpl (Palacios *et al.*, Cell (1985) 41:727-734; GenBank Accession NO. NM\_005373), cynomolgus monkey Mpl (SEQ ID NO: 157), and mouse Mpl (GenBank  
5 Accession NO. NM\_010823) are already known.

[0058]

In addition, the present invention includes antibodies whose binding activities to soluble Mpl are  $KD = 10^{-6}$  M or lower, preferably  $KD = 10^{-7}$  M or lower, and more preferably  $KD = 10^{-8}$  M or lower.

10 [0059]

In the present invention, whether the binding activity of an antibody to soluble recombinant Mpl is  $KD = 10^{-6}$  M or lower can be determined by methods known to those skilled in the art. For example, the activity can be determined using surface plasmon resonance with Biacore. Specifically, soluble MPL-Fc protein is immobilized onto sensor chips. Reaction  
15 rate constant can be determined by assessing the interaction between the antibody and the soluble Mpl-Fc protein. The binding activity can be evaluated by ELISA (enzyme-linked immunosorbent assays), EIA (enzyme immunoassays), RIA (radio immunoassays), or fluorescent antibody techniques. For example, in enzyme immunoassays, a sample containing a test antibody, such as purified antibody or culture supernatant of cells producing the test antibody,  
20 is added to a plate coated with an antigen to which the test antibody can bind. After incubating the plate with a secondary antibody labeled with an enzyme such as alkaline phosphatase, the plate is washed and an enzyme substrate such as p-nitrophenyl phosphate is added. The antigen-binding activity can then be evaluated by determining the absorbance.

[0060]

25 There is no specific limitation as to the upper limit of the binding activity; for example, the upper limit may be set within a technically feasible range by those skilled in the art. However, the technically feasible range may expand with the advancement of technology.

[0061]

In an embodiment, the preferred antibodies of the present invention include antibodies  
30 recognizing epitopes that are recognized by any one of the antibodies indicated in (I) to (XII) below. The antibody of any one of (I) to (XII) is preferably a minibody.

[0062]

(I)

Antibody comprising a VH that has CDR1, 2, and 3 comprising the amino acid  
35 sequences according to SEQ ID NOs in any one of (1) to (22) indicated below (name of each antibody and the H chain CDR contained in the antibody are indicated inside the parentheses):

- (1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3),
- (2) SEQ ID NOs: 6, 7, and 8 (VA130: H chain CDR1, 2, and 3),
- (3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3),
- (4) SEQ ID NOs: 12, 13, and 14 (VB17B: H chain CDR1, 2, and 3),
- 5 (5) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3),
- (6) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3),
- (7) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3),
- (8) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3),
- (9) SEQ ID NOs: 27, 28, and 29 (VB8B: H chain CDR1, 2, and 3),
- 10 (10) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3),
- (11) SEQ ID NOs: 33, 34, and 35 (VB14B: H chain CDR1, 2, and 3),
- (12) SEQ ID NOs: 36, 37, and 38 (VB22B: H chain CDR1, 2, and 3),
- (13) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3),
- (14) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3),
- 15 (15) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3),
- (16) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3),
- (17) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3),
- (18) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3),
- (19) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3),
- 20 (20) SEQ ID NOs: 231, 233, and 235 (hVB22B p-z: H chain CDR1, 2, and 3),
- (21) SEQ ID NOs: 266, 268, and 270 (hVB22B g-e: H chain CDR1, 2, and 3),
- (22) SEQ ID NOs: 280, 282, and 284 (hVB22B e: H chain CDR1, 2, and 3).

[0063]

(II)

- 25 Antibody comprising a VL which has CDR1, 2, and 3 comprising the amino acid sequences according to SEQ ID NOs in any one of (1) to (21) indicated below (name of each antibody and the L chain CDR in the antibody are indicated inside the parentheses):

- (1) SEQ ID NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),
- (2) SEQ ID NOs: 63, 64, and 65 (VA130: L chain CDR1, 2, and 3),
- 30 (3) SEQ ID NOs: 66, 67, and 68 (VA259: L chain CDR1, 2, and 3),
- (4) SEQ ID NOs: 69, 70, and 71 (VB17B: L chain CDR1, 2, and 3),
- (5) SEQ ID NOs: 72, 73, and 74 (VB12B: L chain CDR1, 2, and 3),
- (6) SEQ ID NOs: 75, 76, and 77 (VB140: L chain CDR1, 2, and 3)
- (7) SEQ ID NOs: 78, 79, and 80 (VB33: L chain CDR1, 2, and 3),
- 35 (8) SEQ ID NOs: 81, 82, and 83 (VB45B: L chain CDR1, 2, and 3),
- (9) SEQ ID NOs: 84, 85, and 86 (VB8B: L chain CDR1, 2, and 3),

- (10) SEQ ID NOs: 87, 88, and 89 (VB115: L chain CDR1, 2, and 3),
- (11) SEQ ID NOs: 90, 91, and 92 (VB14B: L chain CDR1, 2, and 3),
- (12) SEQ ID NOs: 93, 94, and 95 (VB22B: L chain CDR1, 2, and 3),
- (13) SEQ ID NOs: 96, 97, and 98 (VB16: L chain CDR1, 2, and 3),
- 5 (14) SEQ ID NOs: 99, 100, and 101 (VB157: L chain CDR1, 2, and 3),
- (15) SEQ ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),
- (16) SEQ ID NOs: 105, 106, and 107 (VB51: L chain CDR1, 2, and 3),
- (17) SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),
- (18) SEQ ID NOs: 111, 112, and 113 (AB324: L chain CDR1, 2, and 3),
- 10 (19) SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3),
- (20) SEQ ID NOs: 240, 242, and 244 (hVB22B p-z: L chain CDR1, 2, and 3),
- (21) SEQ ID NOs: 273, 275, and 277 (hVB22B g-e or hVB22B e: L chain CDR1, 2, and 3).

[0064]

(III)

- 15 Antibody comprising a VH that comprises an amino acid sequence of the SEQ ID NO in any one of (1) to (22):

- (1) SEQ ID NO: 124 (VA7: VH),
- (2) SEQ ID NO: 126 (VA130: VH),
- (3) SEQ ID NO: 128 (VA259: VH),
- 20 (4) SEQ ID NO: 130 (VB17B: VH),
- (5) SEQ ID NO: 132 (VB12B: VH),
- (6) SEQ ID NO: 134 (VB140: VH),
- (7) SEQ ID NO: 136 (VB33: VH),
- (8) SEQ ID NO: 138 (VB45B: VH),
- 25 (9) SEQ ID NO: 140 (VB8B: VH),
- (10) SEQ ID NO: 142 (VB115: VH),
- (11) SEQ ID NO: 144 (VB14B: VH),
- (12) SEQ ID NO: 118 (VB22B: VH),
- (13) SEQ ID NO: 146 (VB16: VH),
- 30 (14) SEQ ID NO: 148 (VB157: VH),
- (15) SEQ ID NO: 150 (VB4B: VH),
- (16) SEQ ID NO: 152 (VB51: VH),
- (17) SEQ ID NO: 155 (AB317: VH),
- (18) SEQ ID NO: 159 (AB324: VH),
- 35 (19) SEQ ID NO: 162 (TA136: VH),
- (20) SEQ ID NO: 229 (hVB22B p-z: VH),



(21) SEQ ID NO: 256 (hVB22B g-e: VH),

(22) SEQ ID NO: 262 (hVB22B e: VH).

[0065]

(IV)

5 Antibody comprising a VL that comprises an amino acid sequence of the SEQ ID NO in any one of (1) to (21):

(1) SEQ ID NO: 125 (VA7: VL),

(2) SEQ ID NO: 127 (VA130: VL),

(3) SEQ ID NO: 129 (VA259: VL),

10 (4) SEQ ID NO: 131 (VB17B: VL),

(5) SEQ ID NO: 133 (VB12B: VL),

(6) SEQ ID NO: 135 (VB140: VL),

(7) SEQ ID NO: 137 (VB33: VL),

(8) SEQ ID NO: 139 (VB45B: VL),

15 (9) SEQ ID NO: 141 (VB8B: VL),

(10) SEQ ID NO: 143 (VB115: VL),

(11) SEQ ID NO: 145 (VB14B: VL),

(12) SEQ ID NO: 120 (VB22B: VL),

(13) SEQ ID NO: 147 (VB16: VL),

20 (14) SEQ ID NO: 149 (VB157: VL),

(15) SEQ ID NO: 151 (VB4B: VL),

(16) SEQ ID NO: 153 (VB51: VL),

(17) SEQ ID NO: 157 (AB317: VL),

(18) SEQ ID NO: 161 (AB324: VL),

25 (19) SEQ ID NO: 163 (TA136: VL),

(20) SEQ ID NO: 238 (hVB22B p-z: VL),

(21) SEQ ID NO: 258 (hVB22B g-e: VL or hVB22B e: VL).

[0066]

(V)

30 Antibody comprising a VH and VL according to any one of (1) to (22):

(1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3); SEQ ID NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),

(2) SEQ ID NOs: 6, 7, and 8 (VA130: H chain CDR1, 2, and 3), SEQ ID NOs: 63, 64, and 65 (VA130: L chain CDR1, 2, and 3),

35 (3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3); SEQ ID NOs: 66, 67, and 68 (VA259: L chain CDR1, 2, and 3),

- (4) SEQ ID NOs: 12, 13, and 14 (VB17B: H chain CDR1, 2, and 3); SEQ ID NOs: 69, 70, and 71 (VB17B: L chain CDR1, 2, and 3),
- (5) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3); SEQ ID NOs: 72, 73, and 74 (VB12B: L chain CDR1, 2, and 3),
- 5 (6) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3); SEQ ID NOs: 75, 76, and 77 (VB140: L chain CDR1, 2, and 3),
- (7) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3); SEQ ID NOs: 78, 79, and 80 (VB33: L chain CDR1, 2, and 3),
- (8) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3); SEQ ID NOs: 81, 82, and 10 83 (VB45B: L chain CDR1, 2, and 3),
- (9) SEQ ID NOs: 27, 28, and 29 (VB8B: H chain CDR1, 2, and 3); SEQ ID NOs: 84, 85, and 86 (VB8B: L chain CDR1, 2, and 3),
- (10) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3); SEQ ID NOs: 87, 88, and 89 (VB115: L chain CDR1, 2, and 3),
- 15 (11) SEQ ID NOs: 33, 34, and 35 (VB14B: H chain CDR1, 2, and 3); SEQ ID NOs: 90, 91, and 92 (VB14B: L chain CDR1, 2, and 3),
- (12) SEQ ID NOs: 36, 37, and 38 (VB22B: H chain CDR1, 2, and 3); SEQ ID NOs: 93, 94, and 95 (VB22B: L chain CDR1, 2, and 3),
- (13) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3); SEQ ID NOs: 96, 97, and 98 20 (VB16: L chain CDR1, 2, and 3),
- (14) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3); SEQ ID NOs: 99, 100, and 101 (VB157: L chain CDR1, 2, and 3),
- (15) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3); SEQ ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),
- 25 (16) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3); SEQ ID NOs: 105, 106, and 107 (VB51: L chain CDR1, 2, and 3),
- (17) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3); SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),
- (18) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3); SEQ ID NOs: 111, 112, 30 and 113 (AB324: L chain CDR1, 2, and 3),
- (19) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3); SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3),
- (20) SEQ ID NOs: 231, 233, and 235 (hVB22B p-z: H chain CDR1, 2, and 3); SEQ ID NOs: 240, 242, and 244 (hVB22B p-z: L chain CDR1, 2, and 3),
- 35 (21) SEQ ID NOs: 266, 268, and 270 (hVB22B g-e: H chain CDR1, 2, and 3); SEQ ID NOs: 273, 275, and 277 (hVB22B g-e: L chain CDR1, 2, and 3),

(22) SEQ ID NOs: 280, 282, and 284 (hVB22B e: H chain CDR1, 2, and 3); SEQ ID NOs: 273, 275, and 277 (hVB22B e: L chain CDR1, 2, and 3).

[0067]

(VI)

5 Antibody comprising a VH and a VL that comprise the amino acid sequences according to SEQ ID NOs in any one of (1) to (22) indicated below:

- (1) SEQ ID NO: 124 (VA7: VH), SEQ ID NO: 125 (VA7: VL),
- (2) SEQ ID NO: 126 (VA130: VH), SEQ ID NO: 127 (VA130: VL),
- (3) SEQ ID NO: 128 (VA259: VH), SEQ ID NO: 129 (VA259: VL),
- 10 (4) SEQ ID NO: 130 (VB17B: VH), SEQ ID NO: 131 (VB17B: VL),
- (5) SEQ ID NO: 132 (VB12B: VH), SEQ ID NO: 133 (VB12B: VL),
- (6) SEQ ID NO: 134 (VB140: VH), SEQ ID NO: 135 (VB140: VL),
- (7) SEQ ID NO: 136 (VB33: VH), SEQ ID NO: 137 (VB33: VL),
- (8) SEQ ID NO: 138 (VB45B: VH), SEQ ID NO: 139 (VB45B: VL),
- 15 (9) SEQ ID NO: 140 (VB8B: VH), SEQ ID NO: 141 (VB8B: VL),
- (10) SEQ ID NO: 142 (VB115: VH), SEQ ID NO: 143 (VB115: VL),
- (11) SEQ ID NO: 144 (VB14B: VH), SEQ ID NO: 145 (VB14B: VL),
- (12) SEQ ID NO: 118 (VB22B: VH), SEQ ID NO: 120 (VB22B: VL),
- (13) SEQ ID NO: 146 (VB16: VH), SEQ ID NO: 147 (VB16: VL),
- 20 (14) SEQ ID NO: 148 (VB157: VH), SEQ ID NO: 149 (VB157: VL),
- (15) SEQ ID NO: 150 (VB4B: VH), SEQ ID NO: 151 (VB4B: VL),
- (16) SEQ ID NO: 152 (VB51: VH), SEQ ID NO: 153 (VB51: VL),
- (17) SEQ ID NO: 155 (AB317: VH), SEQ ID NO: 157 (AB317: VL),
- (18) SEQ ID NO: 159 (AB324: VH), SEQ ID NO: 161 (AB324: VL),
- 25 (19) SEQ ID NO: 162 (TA136: VH), SEQ ID NO: 163 (TA136: VL),
- (20) SEQ ID NO: 229 (hVB22B p-z: VH), SEQ ID NO: 238 (hVB22B p-z: VL),
- (21) SEQ ID NO: 256 (hVB22B g-e: VH), SEQ ID NO: 258 (hVB22B g-e: VL),
- (22) SEQ ID NO: 262 (hVB22B e: VH), SEQ ID NO: 258 (hVB22B e: VL).

[0068]

30 (VII)

Antibody comprising the amino acid sequence of SEQ ID NO: 122 (VB22B: scFv).

[0069]

(VIII)

35 Humanized antibody comprising an amino acid sequence according to any one of SEQ ID NO: 2 (hVB22B p-z: sc(Fv)<sub>2</sub>), SEQ ID NO: 254 (hVB22B g-e: sc(Fv)<sub>2</sub>), and SEQ ID NO: 260 (hVB22B e: sc(Fv)<sub>2</sub>).



[0070]

(IX)

Antibody comprising a VH which has FR1, 2, 3, and 4 comprising amino acid sequences according to SEQ ID NOs in any one of (1) to (3) indicated below:

- 5 (1) SEQ ID NOs: 230, 232, 234, and 236 (hVB22B p-z: H chain FR1, 2, 3, and 4),  
(2) SEQ ID NOs: 265, 267, 269, and 271 (hVB22B g-e: H chain FR1, 2, 3, and 4),  
(3) SEQ ID NOs: 279, 281, 283, and 285 (hVB22B e: H chain FR1, 2, 3, and 4).

[0071]

(X)

- 10 Antibody comprising a VL which has FR1, 2, 3 and 4 comprising amino acid sequences according to SEQ ID NOs in any one of (1) or (2) indicated below:

- (1) SEQ ID NOs: 239, 241, 243, and 245 (hVB22B p-z: L chain FR1, 2, 3, and 4),  
(2) SEQ ID NOs: 272, 274, 276, and 278 (hVB22B g-e or hVB22B e: L chain FR1, 2, 3, and 4).

[0072]

15 (XI)

Antibody comprising VH and VL according to any one of (1) to (3) indicated below:

- (1) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 230, 232, 234, and 236, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 239, 241, 243, and 245, respectively;  
20 (2) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 265, 267, 269, and 271, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively;  
(3) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 279, 281, 283, and 285, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences  
25 of SEQ ID NOs: 272, 274, 276, and 278, respectively.

[0073]

(XII)

Antibody comprising the amino acid sequence of SEQ ID NO: 264 (VB22B: sc(Fv)<sub>2</sub>).

[0074]

- 30 An antibody comprising an amino acid sequence of any one of (I) to (XII) indicated above, in which one or more amino acids have been substituted, deleted, added, and/or inserted, wherein the antibody has activity equivalent to that of the antibody of any one of (I) to (XII).

[0075]

- Herein, the phrase “functionally equivalent” means that an antibody of interest has a  
35 biological or biochemical activity comparable to that of an antibody of the present invention. Such activities include, for example, binding activities and agonistic activities.

[0076]

Methods for preparing polypeptides functionally equivalent to a certain polypeptide are well known to those skilled in the art, and include methods of introducing mutations into polypeptides. For example, those skilled in the art can prepare an antibody functionally  
5 equivalent to the antibodies of the present invention by introducing appropriate mutations into the antibody using site-directed mutagenesis (Hashimoto-Gotoh, T. *et al.* Gene (1995) 152: 271-275; Zoller, MJ, and Smith, M. Methods Enzymol. (1983) 100: 468-500; Kramer, W. *et al.*, Nucleic Acids Res. (1984) 12: 9441-9456; Kramer, W. and Fritz HJ, Methods Enzymol. (1987) 154: 350-367; Kunkel, TA, Proc. Natl. Acad. Sci. USA. (1985) 82: 488-492; Kunkel, Methods  
10 Enzymol. (1988) 85: 2763-2766), or such. Amino acid mutations may occur naturally. Thus, the present invention also comprises antibodies functionally equivalent to the antibodies of the present invention and comprising the amino acid sequences of these antibodies, in which one or more amino acids is mutated. Generally, the number of amino acids that are mutated is 50 amino acids or less, preferably 30 or less, more preferably 10 or less (for example, five amino  
15 acids or less).

[0077]

An amino acid is preferably substituted for a different amino acid(s) that allows the properties of the amino acid side-chain to be conserved. Examples of amino acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids  
20 (R, D, N, C, E, Q, G, H, K, S, and T), amino acids comprising the following side chains: aliphatic side chains (G, A, V, L, I, and P); hydroxyl-containing side chains (S, T, and Y); sulfur-containing side chains (C and M); carboxylic acid- and amide-containing side chains (D, N, E, and Q); basic side chains (R, K, and H); aromatic ring-containing side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses).

25 [0078]

A polypeptide comprising a modified amino acid sequence, in which one or more amino acid residues is deleted, added, and/or replaced with other amino acids, is known to retain its original biological activity (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA (1984) 81: 5662-5666; Zoller, M. J. & Smith, M. Nucleic Acids Research (1982) 10: 6487-6500; Wang, A. *et al.*,  
30 Science 224: 1431-1433; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA (1982) 79: 6409-6413).

[0079]

Fusion proteins containing antibodies that comprise the amino acid sequence of an antibody of the present invention, in which two or more amino acid residues have been added,  
35 are included in the present invention. The fusion protein results from a fusion between one of the above antibodies and a second peptide or protein, and is included in the present invention.

The fusion protein can be prepared by ligating a polynucleotide encoding an antibody of the present invention and a polynucleotide encoding a second peptide or polypeptide in frame, inserting this into an expression vector, and expressing the fusion construct in a host. Some techniques known to those skilled in the art are available for this purpose. The partner peptide or polypeptide to be fused with an antibody of the present invention may be a known peptide, for example, FLAG (Hopp, T. P. *et al.*, BioTechnology (1988) 6: 1204-1210), 6x His consisting of six His (histidine) residues, 10x His, influenza hemagglutinin (HA), human c-myc fragment, VSV-GP fragment, p18HIV fragment, T7-tag, HSV-tag, E-tag, SV40 T antigen fragment, Ick tag,  $\alpha$ -tubulin fragment, B-tag, Protein C fragment. Other partner polypeptides to be fused with the antibodies of the present invention include, for example, GST (glutathione-S-transferase), HA (influenza hemagglutinin), immunoglobulin constant region,  $\beta$ -galactosidase, and MBP (maltose-binding protein). A polynucleotide encoding one of these commercially available peptides or polypeptides can be fused with a polynucleotide encoding an antibody of the present invention. The fusion polypeptide can be prepared by expressing the fusion construct.

[0080]

As described below, the antibodies of the present invention may differ in amino acid sequence, molecular weight, isoelectric point, presence/absence of sugar chains, and conformation depending on the cell or host producing the antibody, or purification method. However, a resulting antibody is included in the present invention, as long as it is functionally equivalent to an antibody of the present invention. For example, when an antibody of the present invention is expressed in prokaryotic cells, for example *E. coli*, a methionine residue is added to the N terminus of the original antibody amino acid sequence. Such antibodies are included in the present invention.

[0081]

An antibody that recognizes an epitope recognized by the antibody according to any one of (I) to (XII) indicated above is expected to have a high agonistic activity. Such antibodies can be prepared by methods known to those skilled in the art. The antibody can be prepared by, for example, determining the epitope recognized by the antibody according to any one of (I) to (XII) by conventional methods, and using a polypeptide comprising one of the epitope amino acid sequences as an immunogen. Alternatively, the antibody can be prepared by determining the epitopes of conventionally prepared antibodies and selecting an antibody that recognizes the epitope recognized by an antibody of any one of (I) to (XII).

[0082]

In the present invention, a particularly preferred antibody is an antibody that recognizes the epitope recognized by the antibody comprising the amino acid sequence of SEQ ID NO: 2. The antibody comprising the amino acid sequence of SEQ ID NO: 2 is predicted to recognize the

region from Glu 26 to Leu 274, preferably the region from Ala 189 to Gly 245, more preferably the region from Gln 213 to Ala 231 of human Mpl. Thus, antibodies recognizing the region of amino acids 26 to 274, or amino acids 189 to 245, or amino acids 213 to 231 of human Mpl are also included in the present invention.

5 [0083]

Antibodies recognizing regions of amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) can be obtained by methods known to those skilled in the art. Such antibodies can be prepared by, for example, using a peptide comprising amino acids 26 to 274, amino acids 189 to 245, or amino  
10 acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) as an immunogen. Alternatively, such antibodies can be prepared by determining the epitope of a conventionally prepared antibody and selecting an antibody that recognizes the same epitope recognized by an antibody of the present invention.

[0084]

15 The present invention provides antibodies described above in (I) to (XII). In an embodiment of the present invention, a preferred antibody is the one shown in (V), a more preferred antibody is the one shown in (VI), and a still more preferred is the one shown in (VIII).

[0085]

The present invention also provides vectors comprising polynucleotides encoding the  
20 antibodies of the present invention, or polynucleotides which hybridize under stringent conditions to the polynucleotides of the present invention and encode antibodies having activities equivalent to those of the antibodies of the present invention. The polynucleotides of the present invention are polymers comprising multiple bases or base pairs of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA), and are not particularly limited, as long as they encode  
25 the antibodies of the present invention. They may also contain non-natural nucleotides. The polynucleotides of the present invention can be used to express antibodies using genetic engineering techniques. The polynucleotides of this invention can also be used as probes in the screening of antibodies functionally equivalent to the antibodies of the present invention. Specifically, DNAs that hybridize under stringent conditions to a polynucleotide encoding an  
30 antibody of the present invention, and encode antibodies having activity equivalent to those of the antibodies of the present invention can be obtained by techniques such as hybridization and gene amplification (for example, PCR), using a polynucleotide of the present invention or a portion thereof as a probe. Such DNAs are also included in the polynucleotides of the present invention. Hybridization techniques are well known to those skilled in the art (Sambrook, J *et al.*, Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989). Such  
35 hybridization conditions include, for example, conditions of low stringency. Examples of



conditions of low stringency include post-hybridization washing in 0.1x SSC and 0.1% SDS at 42°C, and preferably in 0.1x SSC and 0.1% SDS at 50°C. More preferable hybridization conditions include those of high stringency. Highly stringent conditions include, for example, washing in 5x SSC and 0.1% SDS at 65°C. In these conditions, the higher the temperature, the higher the expectation of efficiently obtaining polynucleotides with a high homology. However, several factors, such as temperature and salt concentration, can influence hybridization stringency, and those skilled in the art can suitably select these factors to accomplish similar stringencies.

[0086]

Antibodies that are encoded by polynucleotides obtained by the hybridization and gene amplification techniques, and are functionally equivalent to the antibodies of the present invention generally exhibit high homology to the antibodies of the this invention at the amino acid level. The antibodies of the present invention include antibodies that are functionally equivalent to the antibodies of the present invention, and exhibit high amino acid sequence homology to the antibodies of this invention. The term "high homology" generally means identity at the amino acid level of at least 50% or higher, preferably 75% or higher, more preferably 85% or higher, still more preferably 95% or higher. Polypeptide homology can be determined by the algorithm described in the report: Wilbur, W. J. and Lipman, D. J. Proc. Natl. Acad. Sci. USA (1983) 80: 726-730.

[0087]

When *E. coli* is used as a host, there is no particular limitation as to the type of vector of the present invention, as long as the vector contains an "ori" responsible for its replication in *E. coli* and a marker gene. The "ori" ensures the amplification and mass production of the vector in *E. coli* (for example, JM109, DH5α, HB101, and XL1Blue). The marker gene is used to select the *E. coli* transformants (for example, a drug resistance gene selected by an appropriate drug such as ampicillin, tetracycline, kanamycin, and chloramphenicol). The vectors include, for example, M13 vectors, pUC vectors, pBR322, pBluescript, and pCR-Script. In addition to the above vectors, for example, pGEM-T, pDIRECT, and pT7 can also be used for the subcloning and excision of cDNAs.

[0088]

An expression vector is especially useful for the type of vectors of the present invention. When an expression vector is expressed, for example, in *E. coli*, it should have the above characteristics in order to be amplified in *E. coli*. Additionally, when *E. coli*, such as JM109, DH5α, HB101, or XL1-Blue are used as the host cell, the vector preferably has a promoter, for example, lacZ promoter (Ward *et al.* Nature (1989) 341: 544-546; FASEB J. (1992) 6: 2422-2427), araB promoter (Better *et al.* Science (1988) 240: 1041-1043), or T7 promoter, that



allows efficient expression of the desired gene in *E. coli*. Other examples of the vectors include pGEX-5X-1 (Pharmacia), "QIAexpress system" (QIAGEN), pEGFP, and pET (where BL21, a strain expressing T7 RNA polymerase, is preferably used as the host).

[0089]

Furthermore, the vector may comprise a signal sequence for polypeptide secretion. When producing polypeptides into the periplasm of *E. coli*, the *pelB* signal sequence (Lei, S. P. *et al.* J. Bacteriol. (1987) 169: 4379) may be used as a signal sequence for polypeptide secretion. For example, calcium chloride methods or electroporation methods may be used to introduce the vector into a host cell.

[0090]

In addition to *E. coli*, expression vectors derived from mammals (e.g., pCDNA3 (Invitrogen), pEGF-BOS (Nucleic Acids Res. (1990) 18 (17): 5322), pEF, pCDM8), insect cells (e.g., "Bac-to-BAC baculovirus expression system" (GIBCO-BRL), pBacPAK8), plants (e.g., pMH1, pMH2), animal viruses (e.g., pHSV, pMV, pAdexLcw), retroviruses (e.g., pZIPneo), yeasts (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and *Bacillus subtilis* (e.g., pPL608, pKTH50) may also be used as a vector of the present invention.

[0091]

In order to express proteins in animal cells such as CHO, COS, and NIH3T3 cells, the vector preferably has a promoter necessary for expression in such cells, for example, an SV40 promoter (Mulligan *et al.*, Nature (1979) 277: 108), MMLV-LTR promoter, EF1 $\alpha$  promoter (Mizushima *et al.*, Nucleic Acids Res. (1990) 18: 5322), CMV promoter, etc.). It is even more preferable that the vector also carries a marker gene for selecting transformants (for example, a drug-resistance gene selected by a drug such as neomycin and G418). Examples of vectors with such characteristics include pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13, and such.

[0092]

In addition, to stably express a gene and amplify the gene copy number in cells, CHO cells that are defective in the nucleic acid synthesis pathway are introduced with a vector containing a DHFR gene (for example, pCHOI) to compensate for the defect, and the copy number is amplified using methotrexate (MTX). Alternatively, a COS cell, which carries an SV40 T antigen-expressing gene on its chromosome, can be transformed with a vector containing the SV40 replication origin (for example, pcD) for transient gene expression. The replication origin may be derived from polyoma virus, adenovirus, bovine papilloma virus (BPV), and such. Furthermore, to increase the gene copy number in host cells, the expression vector may contain, as a selection marker, aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, *E. coli* xanthine guanine phosphoribosyl transferase (Ecogpt) gene,

dihydrofolate reductase (dhfr) gene, and such.

[0093]

Next, the vector is introduced into a host cell. The host cells into which the vector is introduced are not particularly limited, for example, *E. coli* and various animal cells are available for this purpose. The host cells may be used, for example, as a production system to produce and express the antibodies of the present invention. *In vitro* and *in vivo* production systems are available for polypeptide production systems. Production systems that use eukaryotic cells or prokaryotic cells are examples of *in vitro* production systems.

[0094]

Eukaryotic cells that can be used are, for example, animal cells, plant cells, and fungal cells. Known animal cells include: mammalian cells, for example, CHO (J. Exp. Med. (1995) 108: 945), COS, 3T3, myeloma, BHK (baby hamster kidney), HeLa, Vero, amphibian cells such as *Xenopus laevis* oocytes (Valle, *et al.*, Nature (1981) 291: 358-340), or insect cells (e.g., Sf9, Sf21, and Tn5). In the present invention, CHO-DG44, CHO-DXB11, COS7 cells, and BHK cells can be suitably used. Among animal cells, CHO cells are particularly favorable for large-scale expression. Vectors can be introduced into a host cell by, for example, calcium phosphate methods, the DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, lipofection methods.

[0095]

Plant cells include, for example, *Nicotiana tabacum*-derived cells known as a protein production system. Calluses may be cultured from these cells. Known fungal cells include yeast cells, for example, genus *Saccharomyces* such as *Saccharomyces cerevisiae* and *Saccharomyces pombe*; and filamentous fungi, for example, genus *Aspergillus* such as *Aspergillus niger*.

[0096]

Bacterial cells can be used in the prokaryotic production systems. Examples of bacterial cells include *E. coli* (for example, JM109, DH5 $\alpha$ , HB101 and such); and *Bacillus subtilis*.

[0097]

Next, the above host cells are cultured. Antibodies can be obtained by transforming the cells with a polynucleotide of interest and *in vitro* culturing of these transformants. Transformants can be cultured using known methods. For example, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium for animal cells, and may be used with or without serum supplements such as FBS or fetal calf serum (FCS). Serum-free cultures are also acceptable. The preferred pH is about 6 to 8 during the course of culturing. Incubation is carried out typically at a temperature of about 30 to 40°C for about 15 to 200 hours. Medium is

exchanged, aerated, or agitated, as necessary.

[0098]

On the other hand, production systems using animal or plant hosts may be used as systems for producing polypeptides *in vivo*. For example, a polynucleotide of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant and then recovered. The "hosts" of the present invention includes such animals and plants.

[0099]

Animals to be used for the production system include mammals or insects. Mammals such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser SPECTRUM Biotechnology Applications (1993)). Alternatively, the mammals may be transgenic animals.

[0100]

For example, a polynucleotide of interest is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat  $\beta$ -casein gene. DNA fragments containing the fusion gene are injected into goat embryos, which are then introduced back to female goats. The desired antibody can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their offspring. Appropriate hormones may be administered to increase the volume of milk containing the antibody produced by the transgenic goats (Ebert, K.M. *et al.*, Bio/Technology (1994) 12: 699-702).

[0101]

Insects, such as silkworms, may also be used. Baculoviruses carrying a polynucleotide encoding an antibody of interest can be used to infect silkworms, and the antibody of interest can be obtained from the body fluids (Susumu, M. *et al.*, Nature (1985) 315: 592-594).

[0102]

Plants used in the production system include, for example, tobacco. When tobacco is used, a polynucleotide encoding an antibody of interest is inserted into a plant expression vector, for example, pMON 530, and then the vector is introduced into a bacterium, such as *Agrobacterium tumefaciens*. The bacteria are then used to infect tobacco such as *Nicotiana tabacum*, and the desired antibodies can be recovered from the leaves (Julian K.-C. Ma *et al.*, Eur. J. Immunol. (1994) 24: 131-138).

[0103]

The resulting antibody may be isolated from the inside or outside (such as the medium) of host cells, and purified as a substantially pure and homogenous antibody. Methods are not limited to any specific method and any standard method for isolating and purifying antibodies may be used. Polypeptides may be isolated and purified, by selecting an appropriate

combination of, for example, chromatographic columns, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, recrystallization, and others.

[0104]

5 Chromatographies include, for example, affinity chromatographies, ion exchange chromatographies, hydrophobic chromatographies, gel filtrations, reverse-phase chromatographies, and adsorption chromatographies (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using liquid phase  
10 chromatographies such as HPLC and FPLC. Examples of the affinity chromatography columns include protein A columns and protein G columns. Examples of the proteins A columns include Hyper D, POROS, and Sepharose F. F. (Pharmacia).

[0105]

15 An antibody can be modified freely and peptide portions deleted by treating the antibody with an appropriate protein modifying enzyme before or after antibody purification. Such protein modifying enzymes include, for example, trypsins, chymotrypsins, lysyl endopeptidases, protein kinases, and glucosidases.

[0106]

20 Antibodies that bind to Mpl can be prepared by methods known to those skilled in the art.

For example, monoclonal antibody-producing hybridomas can be essentially generated by known technologies as follows: immunizing animals with Mpl proteins or Mpl-expressing cells as sensitized antigens using conventional immunological methods; fusing the obtained immunocytes with known parental cells by conventional cell fusion methods; and screening for  
25 monoclonal antibody-producing cells by conventional methods.

[0107]

Specifically, monoclonal antibodies can be prepared by the method below.

First, Mpl protein, which is used as a sensitized antigen for preparing antibodies, is prepared by expressing the Mpl gene/amino acid sequence (GenBank accession number:  
30 NM\_005373). More specifically, the gene sequence encoding Mpl is inserted into a known expression vector, which is then transfected into an appropriate host cell. The subject human Mpl protein is purified from the host cell or culture supernatant using known methods.

[0108]

35 The purified Mpl protein is then used as a sensitized antigen. Alternatively, a partial Mpl peptide may be used as a sensitized antigen. In this case, the partial peptide can also be chemically synthesized based on the amino acid sequence of human Mpl.



[0109]

The epitopes of Mpl molecule that are recognized by an anti-Mpl antibody of the present invention are not limited to a particular epitope, and may be any epitope on the Mpl molecule. Thus, any fragment can be used as an antigen for preparing anti-Mpl antibodies of the present invention, as long as the fragment comprises an epitope of the Mpl molecule.

[0110]

There is no limitation as to the type of mammalian species to be immunized with the sensitized antigen. However, a mammal is preferably selected based on its compatibility with the parental cell to be used in cell fusion. Generally, rodents (for example, mice, rats, and hamsters), rabbits, and monkeys can be used.

[0111]

Animals can be immunized with a sensitized antigen by known methods such as a routine method of injecting a sensitized antigen into a mammal intraperitoneally or subcutaneously. Specifically, the sensitized antigen is diluted appropriately with phosphate-buffered saline (PBS), physiological saline and such, and then suspended. An adequate amount of a conventional adjuvant, for example, Freund's complete adjuvant, is mixed with the suspension, as necessary. An emulsion is then prepared for administering to a mammal several times over a 4- to 21-day interval. An appropriate carrier may be used for the sensitized antigen in immunization.

[0112]

A mammal is immunized as described above. After a titer increase of target antibody in the serum is confirmed, immunocytes are collected from the mammal and then subjected to cell fusion. Spleen cells are the preferred immunocytes.

[0113]

Mammalian myeloma cells are used as the parental cells to be fused with the above immunocytes. Preferable myeloma cells to be used include various known cell lines, for example, P3 (P3x63Ag8.653) (Kearney JF, *et al.*, J. Immunol. (1979) 123: 1548-1550), P3x63Ag8U.1 (Yelton DE, *et al.*, Current Topics in Microbiology and Immunology (1978) 81: 1-7), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. (1976) 6: 511-519), MPC-11 (Margulies, D. H. *et al.*, Cell (1976) 8: 405-415), SP2/0 (Shulman, M. *et al.*, Nature (1978) 276: 269-270), FO (deSt. Groth, S. F. *et al.*, J. Immunol. Methods (1980) 35: 1-21), S194 (Trowbridge, I. S., J. Exp. Med. (1978) 148: 313-323), and R210 (Galfre, G. *et al.*, Nature (1979) 277: 131-133).

[0114]

Cell fusions between the immunocytes and the myeloma cells as described above can be essentially carried out using known methods, for example, a method by Kohler and Milstein (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73: 3-46).



[0115]

More specifically, the above-described cell fusions are carried out, for example, in a conventional culture medium in the presence of a cell fusion-promoting agent. The fusion-promoting agents include, for example, polyethylene glycol (PEG) and Sendai virus (HVV). If required, an auxiliary substance such as dimethyl sulfoxide may also be added to improve fusion efficiency.

[0116]

The ratio of immunocytes to myeloma cells may be determined at one's own discretion, preferably, for example, one myeloma cell for every one to ten immunocytes. Culture media to be used for the above cell fusions include, for example, media that are suitable for the growth of the above myeloma cell lines, such as RPMI 1640 media and MEM media, and other conventional culture media used for this type of cell culture. In addition, serum supplements such as fetal calf serum (FCS) may also be used in combination.

[0117]

Cell fusion is carried out as follows. As described above, predetermined amounts of immunocytes and myeloma cells are mixed well in the culture medium. PEG solution (for example, mean molecular weight of about 1,000-6,000) pre-heated to 37°C is added to the cell suspension typically at a concentration of 30% to 60% (w/v), and mixed to produce fused cells (hybridomas). Then, an appropriate culture medium is successively added to the mixture, and the sample is centrifuged to remove supernatant. This treatment is repeated several times to remove the unwanted cell fusion-promoting agent and others that are unfavorable to hybridoma growth.

[0118]

Screening of the resulting hybridomas can be carried out by culturing them in a conventional selective medium, for example, hypoxanthine, aminopterin, and thymidine (HAT) medium. Culturing in the above-described HAT medium is continued for a period long enough (typically, for several days to several weeks) to kill cells (non-fused cells) other than the desired hybridomas. Then, hybridomas are screened for single-cell clones capable of producing the target antibody by conventional limiting dilution methods.

[0119]

In addition to the method for preparing the above-described hybridomas by immunizing non-human animals with antigens, preferred human antibodies having binding activity to Mpl can also be obtained by: sensitizing human lymphocytes with Mpl *in vitro*; and fusing the sensitized lymphocytes with human myeloma cells capable of dividing permanently (see, Japanese Patent Application Kokoku Publication No. (JP-B) H01-59878 (examined, approved Japanese patent application published for opposition)). Alternatively, it is possible to obtain

human antibodies against Mpl from immortalized cells producing anti-Mpl antibodies. In this method, the cells producing anti-Mpl antibodies are prepared by administering Mpl as an antigen to transgenic animals comprising a repertoire of the entire human antibody genes (see, WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602).

5 [0120]

The monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen over long periods of time.

[0121]

10 Monoclonal antibodies can be prepared from the above-described hybridomas by, for example, a routine procedure of culturing the hybridomas and obtaining antibodies from the culture supernatants. Alternatively, monoclonal antibodies can be prepared by injecting the hybridomas into a compatible mammal; growing these hybridomas in the mammal; and obtaining antibodies from the mammal's ascites. The former method is suitable for preparing highly purified antibodies, while the latter is suitable for preparing antibodies on a large scale.

15 [0122]

Recombinant antibodies can also be prepared by: cloning an antibody gene from a hybridoma; inserting the gene into an appropriate vector; introducing the vector into a host; and producing the antibodies by using genetic recombination techniques (see, for example, Vandamme, A. M. *et al.*, Eur. J. Biochem. (1990) 192: 767-775).

20 [0123]

Specifically, an mRNA encoding the variable (V) region of anti-Mpl antibody is isolated from hybridomas producing the anti-Mpl antibodies. For mRNA isolation, total RNAs are first prepared by conventional methods such as guanidine ultracentrifugation methods (Chirgwin, J. M. *et al.*, Biochemistry (1979) 18: 5294-5299), or acid guanidinium thiocyanate-phenol-chloroform (AGPC) methods (Chomczynski, P. *et al.*, Anal. Biochem. (1987) 162: 156-159), and then the target mRNA is prepared using an mRNA Purification Kit (Pharmacia) and such. Alternatively, the mRNA can be directly prepared using the QuickPrep mRNA Purification Kit (Pharmacia).

[0124]

30 A cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase. cDNA synthesis is carried out using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.), or such. Alternatively, cDNA can be synthesized and amplified by the 5'-RACE method (Frohman, M. A. *et al.*, Proc. Natl. Acad. Sci. USA (1988) 85: 8998-9002; Belyavsky, A. *et al.*, Nucleic Acids Res. (1989) 17: 2919-2932) using the 5'-Ampli  
35 FINDER RACE Kit (Clontech) and PCR.

[0125]

Target DNA fragments are purified from the obtained PCR products and then ligated with vector DNAs to prepare recombinant vectors. The vectors are introduced into *E. coli* and such, and colonies are selected for preparing the recombinant vector of interest. The target DNA nucleotide sequence is then confirmed by conventional methods such as the dideoxynucleotide chain termination method.

Once a DNA encoding the V region of target anti-Mpl antibody is obtained, the DNA is inserted into an expression vector which comprises a DNA encoding the constant region (C region) of a desired antibody.

[0126]

The method for producing anti-Mpl antibodies to be used in the present invention typically comprises the steps of: inserting an antibody gene into an expression vector, so that the gene is expressed under the regulation of expression regulatory regions, such as enhancer and promotor; and transforming host cells with the resulting vectors to express antibodies.

[0127]

For expressing the antibody gene, polynucleotides encoding H chain and L chain, respectively, are inserted into separate expression vectors and co-transfected into a host cell. Alternatively, polynucleotides encoding both H chain and L chain are inserted into a single expression vector and transfected into a host cell (see WO 94/11523).

[0128]

The term “agonistic activity” refers to an activity to induce changes in some biological activities through signal transduction into cells and such, due to the binding of an antibody to a receptor antigen. The biological activities include, for example, proliferation-promoting activities, proliferation activities, viability activities, differentiation-inducing activities, differentiation activities, transcriptional activities, membrane transport activities, binding activities, proteolytic activities, phosphorylation/dephosphorylation activities, oxidation/reduction activities, transfer activities, nucleolytic activities, dehydration activities, cell death-inducing activities, and apoptosis-inducing activities, but is not limited thereto.

[0129]

The term “agonistic activity against Mpl” typically refers to the activity of promoting the differentiation of megakaryocytes or their parental hemopoietic stem cells into platelets, or the activity of stimulating platelet proliferation.

Agonistic activity can be assayed by methods known to those skilled in the art. The agonistic activity may be determined using the original activity or a different activity as an indicator.

[0130]

For example, agonistic activity can be determined by a method using cell growth as an

indicator as described in Examples. More specifically, an antibody whose agonistic activity is to be determined is added to cells which proliferate in an agonist-dependent manner, followed by incubation of the cells. Then, a reagent such as WST-8 which shows a coloring reaction at specific wavelengths depending on the viable cell count, is added to the culture and absorbance is measured. The agonistic activity can be determined using the measured absorbance as an indicator.

[0131]

Cells that proliferate in an agonist-dependent manner can also be prepared by methods known to those skilled in the art. For example, when the antigen is a receptor capable of transducing cell growth signals, cells expressing the receptor may be used. Alternatively, when the antigen is a receptor that cannot transduce signals, a chimeric receptor consisting of the intracellular domain of a receptor that transduces cell growth signals and the extracellular domain of a receptor that does not transduce cell growth signals can be prepared for cellular expression. Receptors that transduce cell growth signals include, for example, G-CSF receptors, mpl, neu, GM-CSF receptors, EPO receptors, c-kit, and FLT-3. Cells that can be used to express a receptor include, for example, BaF3, NFS60, FDCP-1, FDCP-2, CTLL-2, DA-1, and KT-3.

[0132]

There is no limitation as to the type of detection indicators to be used for determining agonistic activity, as long as the indicator can monitor quantitative and/or qualitative changes. For example, it is possible to use cell-free assay indicators, cell-based assay indicators, tissue-based assay indicators, and *in vivo* assay indicators. Indicators that can be used in cell-free assays include enzymatic reactions, quantitative and/or qualitative changes in proteins, DNAs, or RNAs. Such enzymatic reactions include, for example, amino acid transfers, sugar transfers, dehydrations, dehydrogenations, and substrate cleavages. Alternatively, protein phosphorylations, dephosphorylations, dimerizations, multimerizations, hydrolyses, dissociations and such; DNA or RNA amplifications, cleavages, and extensions can be used as the indicator in cell-free assays. For example, protein phosphorylations downstream of a signal transduction pathway may be used as a detection indicator. Alterations in cell phenotype, for example, quantitative and/or qualitative alterations in products, alterations in growth activity, alterations in cell number, morphological alterations, or alterations in cellular properties, can be used as the indicator in cell-based assays. The products include, for example, secretory proteins, surface antigens, intracellular proteins, and mRNAs. The morphological alterations include, for example, alterations in dendrite formation and/or dendrite number, alteration in cell flatness, alteration in cell elongation/axial ratio, alterations in cell size, alterations in intracellular structure, heterogeneity/homogeneity of cell populations, and alterations in cell density. Such



morphological alterations can be observed under a microscope. Cellular properties to be used as the indicator include anchor dependency, cytokine-dependent response, hormone dependency, drug resistance, cell motility, cell migration activity, pulsatory activity, and alteration in intracellular substances. Cell motility includes cell infiltration activity and cell migration activity. The alterations in intracellular substances include, for example, alterations in enzyme activity, mRNA levels, levels of intracellular signaling molecules such as  $\text{Ca}^{2+}$  and cAMP, and intracellular protein levels. When a cell membrane receptor is used, alterations in the cell proliferating activity induced by receptor stimulation can be used as the indicator. The indicators to be used in tissue-based assays include functional alterations adequate for the subject tissue. In *in vivo* assays, alterations in tissue weight, alterations in the blood system (for example, alterations in blood cell counts, protein contents, or enzyme activities), alterations in electrolyte levels, and alterations in the circulating system (for example, alterations in blood pressure or heart rate).

[0133]

The methods for measuring such detection indices are not particularly limited. For example, absorbance, luminescence, color development, fluorescence, radioactivity, fluorescence polarization, surface plasmon resonance signal, time-resolved fluorescence, mass, absorption spectrum, light scattering, and fluorescence resonance energy transfer may be used. These measurement methods are known to those skilled in the art and may be selected appropriately depending on the purpose. For example, absorption spectra can be obtained by using a conventional photometer, plate reader, or such; luminescence can be measured with a luminometer or such; and fluorescence can be measured with a fluorometer or such. Mass can be determined with a mass spectrometer. Radioactivity can be determined with a device such as a gamma counter depending on the type of radiation. Fluorescence polarization can be measured with BEACON (TaKaRa). Surface plasmon resonance signals can be obtained with BIACORE. Time-resolved fluorescence, fluorescence resonance energy transfer, or such can be measured with ARVO or such. Furthermore, a flow cytometer can also be used for measuring. It is possible to use one of the above methods to measure two or more different types of detection indices. A greater number of detection indices may also be examined by using two or more measurement methods simultaneously and/or consecutively. For example, fluorescence and fluorescence resonance energy transfer can be measured at the same time with a fluorometer.

[0134]

The present invention provides pharmaceutical compositions comprising antibodies of this invention. The pharmaceutical compositions comprising antibodies of the present invention are useful for treating and/or preventing thrombocytopenia and such. Time required



for the platelet count to recover to the normal level can be shortened by administering an antibody of the present invention after donation of platelet components. The amount of platelet components at the time of blood collection can be increased by pre-administering an antibody of the present invention.

5 [0135]

When used as pharmaceutical compositions, the antibodies of the present invention can be formulated by methods known to those skilled in the art. For example, the antibodies can be administered parenterally by injection of a sterile solution or suspension in water or other pharmaceutically acceptable solvents. For example, the antibodies can be formulated by  
10 appropriately combining with pharmaceutically-acceptable carriers or solvents, specifically, sterile water or physiological saline, vegetable oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binding agents, and such, and mixing at a unit dosage and form required by accepted pharmaceutical implementations. In such formulations, the amount of the thus obtained active ingredient should be within the  
15 required range.

[0136]

A sterile composition to be injected can be formulated using a vehicle such as distilled water used for injection, according to standard protocols.

Aqueous solutions used for injections include, for example, physiological saline and  
20 isotonic solutions comprising glucose or other adjunctive agents such as D-sorbitol, D-mannose, D-mannitol, and sodium chloride. They may also be combined with an appropriate solubilizing agent such as alcohol, specifically, ethanol, polyalcohol such as propylene glycol or polyethylene glycol, or non-ionic detergent such as polysorbate 80<sup>TM</sup> or HCO-50, as necessary.

[0137]

Oil solutions include sesame oils and soybean oils, and can be combined with  
25 solubilizing agents such as benzyl benzoate or benzyl alcohol. Injection solutions may also be formulated with buffers, for example, phosphate buffers or sodium acetate buffers; analgesics, for example, procaine hydrochloride; stabilizers, for example, benzyl alcohol or phenol; or anti-oxidants. The prepared injections are typically aliquoted into appropriate ampules.

30 [0138]

The administration is preferably carried out parenterally, specifically, by injection, intranasal administration, intrapulmonary administration, percutaneous administration, or such. Injections include, for example, intravenous injections, intramuscular injections, intraperitoneal injections, and subcutaneous injections. The injection solutions can be also administered  
35 systemically or locally.

[0139]

The administration methods can be selected properly according to the patient's age, condition, and such. The applied dose of a pharmaceutical composition comprising an antibody or polynucleotide encoding the antibody may be, for example, in the range of 0.0001 to 1,000 mg/kg body weight. Alternatively, the dosage may be, for example, in the range of 0.001 to 100,000 mg/kg body weight. However, the dosage is not restricted to the values described above. The dosage and administration methods depend on the patient's weight, age, and condition, and are appropriately selected by those skilled in the art.

[Examples]

[0140]

The present invention is specifically illustrated below with reference to Examples, but it is not to be construed as being limited thereto.

[0141]

[Example 1] Preparation of anti-human Mpl antibodies

1.1 Establishment of Mpl-expressing BaF3 cell lines

BaF3 cell lines expressing the full-length Mpl gene were established to obtain cell lines that proliferate in a TPO-dependent manner.

A full-length human Mpl cDNA (Palacios, R. *et al.*, Cell (1985) 41: 727-734) (GenBank accession NO. NM\_005373) was amplified by PCR. The cDNA was cloned into a pCOS2 expression vector to construct pCOS2-hMplfull. The expression vector pCOS2 was constructed by removing the DHFR gene expression region from pCHOI (Hirata, Y. *et al.*, FEBS Letter (1994) 356: 244-248), where the expression region of the neomycin resistance gene HEF-VH-gyl (Sato, K. *et al.*, Mol Immunol. (1994) 31: 371-381) is inserted.

[0142]

The cynomolgus monkey Mpl cDNA (SEQ ID NO: 164) was cloned from total RNA extracted from the bone marrow cells of cynomolgus monkey, using a SMART RACE cDNA Amplification Kit (Clontech). The resulting cynomolgus monkey cDNA was inserted into pCOS2 to construct pCOS2-monkeyMplfull.

Then, the full-length mouse Mpl cDNA (GenBank accession NO. NM\_010823) was amplified by PCR, and inserted into pCOS2 to construct pCOS2-mouseMplfull.

[0143]

Each vector (20 µg) prepared as described above was mixed with BaF3 cells ( $1 \times 10^7$  cells/mL) suspended in PBS in Gene Pulser cuvettes. This mixture was then pulsed at 0.33 kV and 950 µFD using a Gene Pulser II (Bio-Rad). The BaF3 cells introduced with the above DNAs by electroporation were added to RPMI 1640 medium (Invitrogen) containing 1 ng/mL mouse interleukin 3 (hereinafter abbreviated as mIL-3; Peprotech), 500 µg/mL Geneticin (Invitrogen), and 10% FBS (Invitrogen), and selected to establish a human Mpl-expressing BaF3

cell line (hereinafter abbreviated as “BaF3-human Mpl”), monkey Mpl-expressing BaF3 cell line (hereinafter abbreviated as BaF3-monkey Mpl), and mouse Mpl-expressing BaF3 cell line (hereinafter abbreviated as “BaF3-mouse Mpl”). Following selection, these cells were cultured and maintained in RPMI 1640 containing 1 ng/mL rhTPO (R&D) and 10% FBS.

[0144]

## 1.2 Establishment of Mpl-expressing CHO cell lines

CHO cell lines expressing the full-length Mpl gene were established to obtain cell lines to be used for assessing binding activity by flow cytometry.

First, the DHFR gene expression site from pCHOI was inserted into pCXN2 (Niwa, H. *et al.*, Gene (1991) 108: 193-199) at the *Hind*III site to prepare a pCXND3 expression vector. The respective Mpl genes were amplified by PCR using pCOS2-hMplfull, pCOS2-monkeyMplfull, and pCOS2-mouseMplfull as templates, and primers with a His-tag sequence. The PCR products were cloned into pCXND3 to construct pCXND3-hMpl-His, pCXND3-monkey Mpl-His, and pCXND3-mouse Mpl-His, respectively.

[0145]

Vectors thus prepared (25 µg each) were mixed with a PBS suspension of CHO-DG44 cells ( $1 \times 10^7$  cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 µFD using Gene Pulser II (Bio-Rad). The CHO cells introduced with these DNAs by electroporation were added to CHO-S-SFMII medium (Invitrogen) containing 500 µg/mL Geneticin and 1x HT (Invitrogen). A human Mpl-expressing CHO cell line (hereinafter abbreviated as “CHO-human Mpl”), monkey Mpl-expressing CHO cell line (hereinafter abbreviated as “CHO-monkey Mpl”), and mouse Mpl-expressing CHO cell line (hereinafter abbreviated as “CHO-mouse Mpl”) were established through selection.

[0146]

## 1.3 Preparation of soluble human Mpl protein

To prepare soluble human Mpl protein, an expression system using insect Sf9 cells for production and secretion of the protein was constructed as described below.

A DNA construct encoding the extracellular region of human Mpl (Gln 26 to Trp 491) with a downstream FLAG tag was prepared. The construct was inserted into a pBACSurf-1 Transfer Plasmid (Novagen) between the *Pst*I and *Sma*I sites to prepare pBACSurf1-hMpl-FLAG. Then, Sf9 cells were transformed with 4 µg of pBACSurf1-hMpl-FLAG using the Bac-N-Blue Transfection Kit (Invitrogen). The culture supernatant was collected after three-day incubation. Recombinant virus was isolated by plaque assays. The prepared virus stock was used to infect Sf9 cells, and the culture supernatant was collected.

[0147]

Soluble human Mpl protein was purified from the obtained culture supernatant as

described below. The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and the adsorbed protein was then eluted with 50 mM Na-phosphate buffer (pH7.2) containing 0.01% (v/v) Tween20 and 500 mM NaCl. After the eluates were loaded onto a FLAG M2-Agarose (Sigma-Aldrich) for adsorption, the protein adsorbed was eluted with 100 mM glycine-HCl buffer (pH3.5) containing 0.01% (v/v) Tween20. Immediately after elution, the fraction obtained was neutralized with 1 M Tris-Cl (pH8.0) and the buffer was exchanged with PBS (-) and 0.01% (v/v) Tween20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "shMpl-FLAG".

[0148]

#### 1.4 Preparation of human Mpl-IgG Fc fusion protein

Human fusion protein Mpl-IgG Fc gene was prepared according to the method by Bennett *et al.* (Bennett, B. D. *et al.*, J. Biol. Chem. (1991) 266: 23060-23067). A nucleotide sequence encoding the extracellular region of human Mpl (Gln 26 to Trp 491) was linked to a nucleotide sequence encoding the Fc region of human IgG- $\gamma$ 1 (a region downstream of Asp 216). A *Bst*EII sequence (amino acids: Val-Thr) was attached to the junction as a fusion linker between these two regions. A 19-amino acid signal peptide derived from human IgG H chain variable region was used as the signal sequence. The resulting human fusion protein Mpl-IgG Fc gene was cloned into pCXND3 to construct pCXND3-hMpl-Fc.

[0149]

The vector thus prepared (25  $\mu$ g) was mixed with a PBS suspension of CHO-DG44 cells ( $1 \times 10^7$  cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25  $\mu$ FD using Gene Pulser II (Bio-Rad). The CHO cells introduced with the DNA by electroporation were added to CHO-S-SFMII medium containing 500  $\mu$ g/mL Geneticin and 1x HT (Invitrogen). shMPL-Fc-expressing CHO cell line (CHO-hMpl-Fc) was then established through selection.

[0150]

Human Mpl-IgG Fc fusion protein was purified from the culture supernatant as described below.

The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and then the adsorbed protein were eluted with 50 mM Na-phosphate buffer (pH7.6) containing 0.01% (v/v) Tween20 and 1 M NaCl. After the eluates were loaded onto a HiTrap protein G HP column (Amersham Biosciences) for adsorption, the adsorbed protein was eluted with 0.1 M glycine-HCl buffer (pH2.7) containing 150 mM NaCl and 0.01% (v/v) Tween20. Immediately after elution, the obtained fraction was neutralized with 1 M Tris-Cl (pH8.0) and the buffer was exchanged with PBS (-) and 0.01% (v/v) Tween20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "hMpl-Fc".



[0151]

#### 1.5 Immunization with shMpl-FLAG or BaF3-human Mpl and hybridoma selection

MRL/MpJUmmCrj-lpr/lpr mice (hereinafter abbreviated as “MRL/lpr mice”; purchased from Charles River, Japan) were immunized; the primary immunization was carried out at eight weeks of age. For every single mouse, an emulsion containing 100 µg of shMPL-FLAG combined with Freund’s complete adjuvant (H37 Ra; Beckton Dickinson), was administered subcutaneously as the primary injection. As a booster injection, an emulsion containing shMPL-FLAG (50 µg per mouse) combined with Freund’s incomplete adjuvant (Beckton Dickinson) was administered subcutaneously. Three mice which have been immunized six times in total were subjected to a final injection of shMPL-FLAG (50 µg per mouse) through the caudal vein. Cell fusion was achieved by mixing the mouse myeloma P3-X63Ag8U1 cells (P3U1; purchased from ATCC) and mouse splenocytes using polyethylene glycol 1500 (Roche Diagnostics). Hybridoma selection in HAT medium began the following day and culture supernatants were obtained. Screening was carried out by ELISA, using immunoplates immobilized with shMpl-FLAG or hMpl-Fc and the assayed cell growth activity of BaF3-human Mpl as an index. In addition, Balb/C mice were immunized eleven times in total by administering BaF3-human Mpl ( $1.0 \times 10^7$  cells per mouse) intraperitoneally over a period of one week to five months. Hybridomas were similarly prepared by cell fusion, and screened using the assayed cell growth activity of BaF3-human Mpl as an index. Positive clones were isolated as single clones by limiting dilution and then cultured in a large scale. The culture supernatants were collected.

[0152]

#### 1.6 Analyses of anti-human Mpl antibodies

Antibody concentrations were determined by carrying out a mouse IgG sandwich ELISA using goat anti-mouse IgG (gamma) (ZYMED) and alkaline phosphatase-goat anti-mouse IgG (gamma) (ZYMED), generating a calibration curve by GraphPad Prism (GraphPad Software; USA), and calculating the antibody concentrations from the calibration curve. Commercially available antibodies of the same isotype were used as standards.

[0153]

Antibody isotypes were determined by antigen-dependent ELISA using isotype-specific secondary antibodies. hMpl-Fc was diluted to 1 µg/mL with a coating buffer (0.1 mM NaHCO<sub>3</sub>, pH9.6) containing 0.02% (w/v) NaN<sub>3</sub>, and then added to ELISA plates. The plates were incubated overnight at 4°C for coating. The plates were blocked with a diluent buffer (50 mM Tris-HCl (pH8.1) containing 1 mM MgCl<sub>2</sub>, 150 mM NaCl, 0.05% (v/v) Tween20, 0.02% (w/v) NaN<sub>3</sub>, 1% (w/v) BSA). After the addition of hybridoma culture supernatants, the plates were allowed to stand at room temperature for 1 hr. After washing with a rinse buffer (0.05% (v/v)



Tween20 in PBS), alkaline phosphatase-labeled isotype-specific secondary antibodies were added to the plates. Then, the plates were allowed to stand at room temperature for 1 hr. Color development was carried out using SIGMA104 (Sigma-Aldrich) diluted to 1 mg/mL with a substrate buffer (50 mM NaHCO<sub>3</sub>, pH9.8) containing 10 mM MgCl<sub>2</sub>, and absorbance was measured at 405 nm using Benchmark Plus (Bio-Rad).

[0154]

The binding activities of an antibody to shMpl-FLAG and hMPL-Fc were determined by ELISA. ELISA plates were coated with 1 µg/mL of purified shMpl-FLAG or hMPL-Fc, and blocked with a diluent buffer. Hybridoma culture supernatants were added to the plates, and the plates were allowed to stand at room temperature for 1 hr. Then, alkaline phosphatase-labeled anti-mouse IgG antibodies (Zymed) were added to the plates. Color development was similarly carried out using the above method. Following a one-hour coloring reaction at room temperature, absorbance was measured at 405 nm and EC<sub>50</sub> values were computed using GraphPad Prism.

[0155]

CHO-human Mpl cells and CHO-monkey Mpl cells were harvested, and suspended in FACS Buffer (1% FBS/ PBS) to a final concentration of 1 x 10<sup>6</sup> cells/mL. The suspensions were aliquoted into Multiscreen (Millipore) at 100 µl/well, and the culture supernatants were removed by centrifugation. Culture supernatants diluted to 5 µg/mL were added to the plates and incubated on ice for 30 min. The cells were washed once with FACS buffer, and incubated on ice for 30 min following the addition of an FITC-labeled anti-mouse IgG antibody (Beckman Coulter). After incubation, the mixture was centrifuged at 500 rpm for 1 min. The supernatants were removed, and then the cells were suspended in 400 µL of FACS buffer. The samples were analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

[0156]

Agonistic activities of an antibody were evaluated using BaF3-human Mpl and BaF3-monkey Mpl which proliferate in a TPO-dependent manner. Cells of each cell line were suspended at 4 x 10<sup>5</sup> cells/ml in RPMI 1640/10% FBS (Invitrogen), and each suspension was aliquoted into a 96-well plate at 60µl/well. A 40-µL aliquot of rhTPO (R&D) and hybridoma culture supernatants prepared at various concentrations was added into each well. The plates were then incubated at 37°C under 5% CO<sub>2</sub> for 24 hr. A 10-µL aliquot of the Cell Count Reagent SF (Nacalai Tesque) was added into each well. After incubation for 2 hr, absorbance was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. EC<sub>50</sub> values were calculated using GraphPad Prism.

The above analysis yielded a total of 163 clones of mouse monoclonal antibodies that bind to human Mpl.

Among the anti-human Mpl antibodies to be described, TA136 was established from mice immunized with BaF-human Mpl and the others were established from mice immunized with shMpl-Flag.

[0157]

#### 1.7 Purification of anti-human Mpl antibodies

Anti-human Mpl antibodies were purified from hybridoma culture supernatants as described below.

After the culture supernatants were loaded onto HiTrap protein G HP columns (Amersham Biosciences) for adsorption, the antibodies were eluted with 0.1 M glycine-HCl (pH2.7). Immediately after elution, the fractions were neutralized with 1 M Tris-Cl (pH9.0), dialyzed against PBS for one day, and the buffer was replaced.

[0158]

#### 1.8 Determination of epitopes for the anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B can be used for Western blotting, a GST-fusion protein containing a partial sequence of human Mpl was constructed for VB22B epitope analysis. MG1 (Gln26 to Trp491) and MG2 (Gln26 to Leu274) regions were each amplified by PCR, and cloned into pGEX-4T-3 (Amersham Biosciences) to be expressed as GST fusion proteins. The resulting plasmid DNAs were transformed into DH5 $\alpha$  to give transformants. A final concentration of 1 mM IPTG was added to the transformants in their logarithmic growth phase to induce the expression of GST fusion proteins. The bacterial cells were harvested after two hours of incubation. The cells were lysed by sonication. The lysates were centrifuged in XL-80 Ultracentrifuge (Beckman, Rotor 70.1Ti) at 35,000 rpm for 30 min. The culture supernatants were removed, and then the fusion proteins were purified using GST Purification Modules (Amersham Biosciences). The samples were separated by 10%-SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was Western blotted with the murine antibody VB22B. VB22B was found to recognize both MG-1 and MG-2, indicating that the VB22B epitope is located in the (Gln26 to Leu274) region.

[0159]

Then, GST fusion proteins containing the respective regions of human Mpl: MG3 (Gln26 to Ala189), MG4 (Gln26 to Pro106), MG5 (Gln26 to Glu259), and MG6 (Gln26 to Gly245) were prepared and analyzed by Western blotting using the same procedure described above. VB22B was found to recognize MG5 and MG6, but not MG3 and MG4. This suggests that the VB22B epitope is located within the (Ala189 to Gly245) region. In addition, GST was fused with MG7 (Gln26 to Ala231) and MG8 (Gln26 to Pro217) to prepare GST fusion

proteins. VB22B recognized MG7 but not MG8, suggesting that the VB22B epitope is located in the (Gln217 to Ala231) region. Furthermore, GST fusion protein containing MG10 (Gln213 to Ala231) was recognized by VB22B, suggesting that the VB22B epitope is located within the limited region of 19 amino acids between Gln213 and Ala231.

[0160]

#### 1.9 Kinetic analyses of the antigen-antibody reaction for anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B binds to soluble recombinant Mpl, kinetic analyses of the antigen-antibody reaction between VB22B IgG and human Mpl-IgG Fc fusion protein were carried out as described in Example 1.4. The Sensor Chip CM5 (Biacore) was placed in Biacore 2000 (Biacore), and human Mpl-IgG Fc fusion protein was immobilized onto the chip by amine-coupling methods. Then, 1.25 to 20  $\mu\text{g/mL}$  of VB22B IgG solution was prepared using HBS-EP Buffer (Biacore), and injected over the chip surface for 2 min to reveal the binding region. Then, HBS-EP Buffer was injected over the chip surface for 2 min to reveal the dissociation region. VB22B IgG bound to the human Mpl-IgG Fc fusion protein on the sensor chip was removed by injecting 10 mM NaOH over the sensor chip for 15 sec, and the chip was recovered. HBS-EP Buffer was used as the running buffer, and the flow rate was 20  $\mu\text{L/min}$ . Using the BIAevaluation Version 3.1 (Biacore) software, the reaction rate constant at each concentration was calculated from the sensorgrams. The dissociation constant ( $K_D$ ) for VB22B IgG was determined to be  $1.67 \pm 0.713 \times 10^{-9}$  M.

[0161]

#### [Example 2] Preparation of single-chain anti-human Mpl antibodies

Among the prepared anti-human Mpl antibodies, 23 types of antibodies, which exhibit higher binding activities and agonistic activities, were selected to construct expression systems for single-chain antibodies using genetic engineering techniques. An exemplary method for constructing a single-chain antibody derived from the anti-human Mpl antibody VB22B is described below.

[0162]

#### 2.1 Cloning of the anti-human Mpl antibody variable region

The variable region was amplified by RT-PCR using total RNA extracted from hybridomas producing anti-human Mpl antibodies. Total RNA was extracted from  $1 \times 10^7$  hybridoma cells using the RNeasy Plant Mini Kit (QIAGEN).

[0163]

A 5'-terminal fragment of the gene was amplified from 1  $\mu\text{g}$  of total RNA by the SMART RACE cDNA Amplification Kit (Clontech), using a synthetic oligonucleotide MHC-IgG2b (SEQ ID NO: 166) complementary to mouse IgG2b constant region or a synthetic oligonucleotide kappa (SEQ ID NO: 167) complementary to mouse  $\kappa$  chain constant region.

Reverse transcription was carried out at 42°C for 1.5 hr.

[0164]

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x Advantage 2 PCR Buffer (Clontech)	5 µL
10x Universal Primer A Mix (Clontech)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (Clontech)	0.2 mM
Advantage 2 Polymerase Mix (Clontech)	1 µL
Reverse transcription product	2.5 µL
Synthetic oligonucleotide, MHC-IgG2b or kappa	10 pmol

[0165]

5 The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 5 sec and 72°C for 3 min;

five cycles of 94°C for 5 sec, 70°C for 10 sec, and 72°C for 3 min;

25 cycles of 94°C for 5 sec, 68°C for 10 sec, and 72°C for 3 min;

10 and final extension was at 72°C for 7 min.

[0166]

The PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and cloned into a pGEM-T Easy Vector (Promega). The nucleotide sequence was then determined using the ABI 3700 DNA Analyzer (Perkin Elmer).

15 [0167]

The nucleotide sequence of cloned VB22B H chain variable region (hereinafter abbreviated as "VB22B-VH") is shown in SEQ ID NO: 117, and its amino acid sequence is shown in SEQ ID NO: 118. The nucleotide sequence of the L chain variable region (hereinafter abbreviated as "VB22B-VL") is shown in SEQ ID NO: 119, and its amino acid sequence is shown in SEQ ID NO: 120.

[0168]

## 2.2 Preparation of expression vectors for anti-human Mpl diabodies

25 The gene encoding VB22B single-chain Fv (hereinafter abbreviated as "VB22B diabody") containing a five-amino acid linker sequence was constructed, by linking a nucleotide sequence encoding a (Gly4Ser)<sub>1</sub> linker to the VB22B-VH-encoding gene at its 3' end and to the VB22B-VL-encoding gene at its 5' end; both of which have been amplified by PCR.

[0169]

30 The VB22B-VH forward primer, 70·115HF, (SEQ ID NO: 168) was designed to contain an *Eco*RI site. The VB22B-VH reverse primer, 33·115HR, (SEQ ID NO: 169) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence



encoding the (Gly4Ser)<sub>1</sub> linker and a nucleotide sequence hybridizing to the DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, 33·115LF, (SEQ ID NO: 170) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly4Ser)<sub>1</sub> linker, and a nucleotide sequence encoding the C terminus of VB22B-VH. The VB22B-VL reverse primer, 33·115LR, (SEQ ID NO: 171) was designed to hybridize to a DNA encoding the C terminus of VB22B-VL and to have a nucleotide sequence encoding a FLAG tag (Asp Tyr Lys Asp Asp Asp Asp Lys/SEQ ID NO: 172) and a *NotI* site.

[0170]

In the first round of PCR, two PCR products: one containing VB22B-VH and a linker sequence, and the other containing VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50  $\mu$ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 $\mu$ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pGEM-T Easy vector comprising VB22B-VH or VB22B-VL gene	10 ng
Synthetic oligonucleotides, 70·115HF and 33·115HR, or 33·115LF and 33·115LR	10 pmol

[0171]

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;  
 five cycles of: 94°C for 15 sec and 72°C for 2 min;  
 five cycles of 94°C for 15 sec and 70°C for 2 min;  
 28 cycles of 94°C for 15 sec and 68°C for 2 min;  
 and final extension was at 72°C for 5 min.

[0172]

After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50  $\mu$ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 $\mu$ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 unit
First-round PCR products (two types)	1 $\mu$ L
Synthetic oligonucleotides, 70·115HF and 33·115LR	10 pmol

[0173]



The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

5 28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0174]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then digested with *Eco*RI and *Not*I (both from TaKaRa).

10 The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pCXND3 to prepare pCXND3-VB22B db.

[0175]

### 2.3 Preparation of expression vectors for anti-human Mpl antibody sc(Fv)<sub>2</sub>

15 To prepare expression plasmids for the modified antibody [sc(Fv)<sub>2</sub>] comprising two units of H chain variable region and two units of L chain variable region derived from VB22B, the above-described pCXND3-VB22B db was modified by PCR using the procedure shown below. The process for constructing the sc(Fv)<sub>2</sub> gene is illustrated in Fig. 1.

[0176]

20 First, PCR method was carried out to amplify (a) the VB22B-VH-encoding gene in which a nucleotide sequence encoding a 15-amino acid linker (Gly<sub>4</sub>Ser)<sub>3</sub> was added to its 3' end; and (b) the VB22B-VL-encoding gene containing the identical linker nucleotide sequence added to its 5' end. The desired construct was prepared by linking these amplified genes. Three new primers were designed in this construction process. The VB22B-VH forward primer, VB22B-fpvu, (primer A; SEQ ID NO: 173) was designed to have an *Eco*RI site at its 5' end and to convert Gln22 and Leu23 of VB22B db into a *Pvu*II site. The VB22B-VH reverse primer, sc-rL15, (primer B; SEQ ID NO: 174) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker, as well as a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, sc-fL15, (primer C; SEQ ID NO: 175) was designed to have a  
25 nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker, and a nucleotide sequence encoding the C terminus of VB22B-VH.

[0177]

35 In the first-round PCR, two PCR products: one comprising VB22B-VH and a linker sequence, and the other comprising VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 $\mu$ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pCXND3-VB22B db	10 ng
Synthetic oligonucleotides, VB22B-fpvu, sc-rL15 or sc-fL15, and 33-115LR (primer D)	10 pmol

[0178]

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

5 five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0179]

10 After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50  $\mu$ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 $\mu$ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
First-round PCR product (two types)	1 $\mu$ L
Synthetic oligonucleotide, 70-115HF and 33-115LR	10 pmol

[0180]

The reaction conditions were:

15 94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

20 [0181]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then digested with *Eco*RI and *Not*I (both from TaKaRa).

The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pBacPAK9 (Clontech) to construct pBacPAK9-scVB22B.

25 [0182]

A fragment to be inserted into the *PvuII* site of pBacPAK9-scVB22B was prepared. Specifically, the fragment has a *PvuII* recognition site at both ends and a nucleotide sequence, in which a gene encoding the VB22B-VH N-terminus is linked, *via* a (Gly<sub>4</sub>Ser)<sub>3</sub> linker-encoding nucleotide sequence, to a gene encoding the amino acid sequence of an N-terminus-deleted VB22B-VH linked to VB22B-VL *via* the (Gly<sub>4</sub>Ser)<sub>3</sub> linker. Two primers were newly designed to prepare the fragment by PCR. The forward primer for the fragment of interest, Fv2-f (primer E; SEQ ID NO: 176), was designed to have a *PvuII* site at its 5' end and a VB22B-VH 5'-end sequence. The reverse primer for the fragment of interest, Fv2-r (primer F; SEQ ID NO: 177), was designed to hybridize to a DNA encoding the C terminus of VB22B-VL, and to have a *PvuII* site, a nucleotide sequence encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker, and a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VH. PCR was carried out using pBacPAK9-scVB22B as a template as described below.

[0183]

The composition of the PCR reaction solution (50  $\mu$ L in total) is shown below.

10x PCR Buffer (TaKaRa)	5 $\mu$ L
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pBacPAK9-scVB22B	10 $\mu$ g
Synthetic oligonucleotide, Fv2-f and Fv2-r	10 pmol

[0184]

The reaction conditions were:

94°C (initial temperature) for 30 sec;  
 five cycles of 94°C for 15 sec and 72°C for 2 min;  
 five cycles of 94°C for 15 sec and 70°C for 2 min;  
 28 cycles of 94°C for 15 sec and 68°C for 2 min;  
 and final extension was at 72°C for 5 min.

[0185]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then cloned into the pGEM-T Easy Vector (Promega).

After sequencing, the plasmid was digested with *PvuII* (TaKaRa), and the fragment of interest was recovered. The recovered fragment was ligated to pBacPAK9-scVB22B pre-digested with *PvuII* (TaKaRa) to construct pBacPAK9-VB22B sc(Fv)<sub>2</sub>. After the resulting vector was digested with *EcoRI* and *NotI* (both from TaKaRa), the fragment of about 1,600 bp was purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The fragment was then cloned into a pCXND3 expression vector to construct pCXND3-VB22B sc(Fv)<sub>2</sub>.

[0186]

## 2.4 Expression of single-chain anti-human Mpl antibody in animal cells

A cell line stably expressing the single-chain antibody was prepared from CHO-DG44 cells as described below. Gene transfer was achieved by electroporation using a Gene Pulser II (Bio-Rad). An expression vector (25  $\mu$ g) and 0.75 mL of CHO-DG44 cells suspended in PBS (1 x 10<sup>7</sup> cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and pulsed at 1.5-kV and 25  $\mu$ FD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in CHO-S-SFMII medium (Invitrogen) containing 500  $\mu$ g/mL Geneticin (Invitrogen). CHO cell lines expressing the single-chain antibody were established through selection. A cell line stably expressing VB22B sc(Fv)<sub>2</sub> and its culture supernatants were obtained by this method.

[0187]

The transient expression of the single-chain antibody was achieved using COS7 cells as described below. An expression vector (10  $\mu$ g) and 0.75 mL of COS7 cells suspended in PBS (1 x 10<sup>7</sup> cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and then pulsed at 1.5-kV and 25  $\mu$ FD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in DMEM/10% FBS medium (Invitrogen). The cells were incubated overnight and then washed with PBS. CHO-S-SFMII medium was added and the cells were cultured for about three days. The culture supernatants for preparing the VB22B diabody were thus prepared.

[0188]

## 2.5 Quantitation of single-chain anti-human Mpl antibodies in culture supernatants

The culture supernatant concentration of the single-chain anti-human Mpl antibody transiently expressed in COS cells was determined using surface plasmon resonance. A sensor chip CM5 (Biacore) was placed in Biacore 2000 (Biacore). ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich) was immobilized onto the chip. An appropriate concentration of sample was injected over the chip surface at a flow rate of 5 mL/sec, and 50 mM diethylamine was used to dissociate the bound antibody. Changes in the mass during sample injection were recorded, and the sample concentration was calculated from the calibration curve prepared using the mass changes of a standard sample. db12E10 (see WO 02/33073 and WO 02/33072) was used as the diabody standard, and 12E10 sc(Fv)<sub>2</sub> which has the same gene structure as that of sc(Fv)<sub>2</sub> was used as the sc(Fv)<sub>2</sub> standard.

[0189]

## 2.6 Purification of anti-human Mpl diabodies and single-chain antibodies

The culture supernatants of VB22B diabody-expressing COS7 cells or CHO cells was loaded onto an Anti-Flag M2 Affinity Gel (Sigma-Aldrich) column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween20. The absorbed



antibodies were eluted with 100 mM glycine-HCl (pH3.5). The fractions eluted were immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography. PBS/0.01% Tween20 was used in the gel filtration chromatography.

5 [0190]

VB22B sc(Fv)<sub>2</sub> was purified from the culture supernatants of VB22B sc(Fv)<sub>2</sub>-expressing COS7 cells or CHO cells under the same conditions used for purifying the diabodies. A large-scale preparation of VB22B sc(Fv)<sub>2</sub> was prepared by loading the CHO cell culture supernatants onto a Macro-Prep Ceramic Hydroxyapatite Type I (Bio-Rad) column equilibrated with a 20 mM phosphate buffer (pH6.8), and eluting the VB22B sc(Fv)<sub>2</sub> in a stepwise manner with 250 mM phosphate buffer (pH6.8). The eluted fraction was concentrated on an ultrafilter, and then fractionated by gel filtration chromatography using a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column, and a fraction corresponding to the molecular weight range of about 40 kD to 70 kD was obtained. The fraction was loaded onto an Anti-Flag M2 Affinity Gel column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween20. The absorbed antibody was eluted with 100 mM glycine-HCl (pH3.5). The eluted fraction was immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography. 20 mM acetate buffer (pH6.0) containing 150 mM NaCl and 0.01% Tween80 was used in the gel filtration chromatography. In each purification step, the presence of the diabody and sc(Fv)<sub>2</sub> in the samples was confirmed by SDS-PAGE and Western blotting using an anti-Flag antibody (Sigma-Aldrich).

[0191]

## 2.7 Binding activity analyses of single-chain anti-human Mpl antibodies by flow cytometry

25 CHO-human Mpl, CHO-monkey Mpl, and CHO-mouse Mpl cells were recovered and suspended in FACS buffer (1% FBS/PBS) to a final concentration of  $1 \times 10^6$  cells/mL. Cell suspensions were aliquoted at 100- $\mu$ L/well into the Multiscreen-HV Filter Plates (Millipore). After centrifugation, the supernatant was removed. An appropriate concentration of diabody or sc(Fv)<sub>2</sub> was added into each well and incubated on ice for 30 min. The cells were washed once with 200  $\mu$ L of FACS buffer, and incubated on ice for 30 min following the addition of 10  $\mu$ g/mL ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich). The cells were then washed once with 200  $\mu$ L of FACS buffer, and a 100x-diluted FITC-labeled anti-mouse IgG antibody (Beckman Coulter) was added to the plate. The plate was incubated on ice for 30 min. After centrifugation, the supernatant was removed. The cells were suspended in 400  $\mu$ L of FACS Buffer, and then analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell



populations.

[0192]

The binding activity of the purified VB22B sc(Fv)<sub>2</sub> to various Mpl molecules expressed in CHO cells was determined (Fig. 2). VB22B sc(Fv)<sub>2</sub> was found to specifically bind to  
5 CHO-human Mpl and CHO-monkey Mpl but not to the host cell CHO or CHO-mouse Mpl. This binding characteristic of VB22B sc(Fv)<sub>2</sub> is comparable to those of VB22B IgG<sub>1</sub> indicating that the antibody binding site remains unaltered by reduction of molecular weight.

[0193]

#### 2.8 Analyses of TPO-like agonistic activity for single-chain anti-human Mpl antibodies

10 TPO-like agonistic activity was assessed using BaF3-human Mpls or BaF3-monkey Mpls that proliferate in a TPO-dependent manner.

Cells from each cell line were washed twice with RPMI 1640/1% FBS (fetal bovine serum) (Invitrogen), and then suspended in RPMI 1640/10% FBS to a concentration of  $4 \times 10^5$  cells/mL. Cell suspensions were aliquoted at 60- $\mu$ L/well into a 96-well plate. Various  
15 concentrations of rhTPO (R&D) and COS7 culture supernatants or purified samples were prepared, and a 40- $\mu$ L aliquot was added into each well. The plates were then incubated at 37°C under 5% CO<sub>2</sub> for 24 hr. Immediately after a 10- $\mu$ L aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added into each well, absorbance was measured at 450 nm (and at 655 nm as a control) using Benchmark Plus. After two hours of incubation,  
20 absorbance was again measured at 450 nm (and at 655 nm as a control). The WST-8 reagent changes colors at 450 nm in a color reaction that reflects the viable cell count. The TPO-like agonistic activity was assessed using the change in absorbance during the two-hour incubation as an index. EC<sub>50</sub> values were computed using GraphPad Prism.

[0194]

25 TPO-like agonistic activity was assayed using the human leukemia cell line M-07e (purchased from DSMZ) which proliferates TPO-dependently. M-07e cells were washed twice with RPMI 1640/1% FBS, and then suspended in RPMI 1640/10% FBS to a concentration of  $5 \times 10^5$  cells/mL. The resulting cell suspension was aliquoted at 50- $\mu$ L/well into a 96-well plate. Various concentrations of rhTPO and COS7 culture supernatants or purified samples were  
30 prepared, and a 50- $\mu$ L aliquot was added into each well. The plates were then incubated at 37°C under 5% CO<sub>2</sub> for 48 hr. Immediately after a 10- $\mu$ L aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added to each well, absorbance of was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. After four hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The TPO-like  
35 agonistic activity was assayed using the change in absorbance during the four-hour incubation as an index.

[0195]

Purified VB22B IgG, VB22B diabody, and VB22B sc(Fv)<sub>2</sub> were assayed for their TPO-like agonistic activities using BaF3-human Mpl, BaF3-monkey Mpl, and M-07e. The results are shown in Figures 3, 4, and 5, respectively. The presence of bivalent antigen-binding domains in a single antibody molecule is essential for its agonistic activity. The distance and angle between two antigen-binding domains can also be important factors (see WO 02/33073 and WO 02/33072). Similar results were obtained for the newly isolated anti-human Mpl antibodies. Specifically, the agonistic activities of VB22B diabody and VB22B sc(Fv)<sub>2</sub> (EC<sub>50</sub> = 61 pM and 27 pM in BaF-human Mpl, respectively) were higher than that of VB22B IgG (EC<sub>50</sub> > 30 nM in BaF-human Mpl), and were equivalent to or higher than that of the naturally-occurring human TPO ligand (EC<sub>50</sub> = 76 pM in BaF-human Mpl). The VB22B diabody activity was lower than that of VB22B sc(Fv)<sub>2</sub>. This suggests that the structure of a single-chain antibody is greatly altered by its molecular shape and the length of the linker sequence, which in turn changes the agonistic activity. Sixteen types of the single-chain anti-human Mpl antibodies were obtained, each exhibiting a high agonistic activity. The amino acid sequences of the H chain and L chain variable regions of the representative antibodies are shown in Figures 6 and 7, respectively.

[0196]

## 2.9 Humanization of single-chain anti-human Mpl antibody

Antibody sequence data for the humanization of VB22B sc(Fv)<sub>2</sub> were obtained from the Kabat Database (<ftp://ftp.ebi.ac.uk/pub/databases/kabat/>), and homology searches were carried out independently for the H chain variable region and the L chain variable region. As a result, the H chain variable region was found to be highly homologous to DN13 (Smithson S. L. *et al.*, Mol Immunol. (1999) 36: 113-124). The L chain variable region was found to be highly homologous to ToP027 (Hougs L. *et al.*, J. Immunol. (1999) 162: 224-237). Humanized antibodies were prepared by inserting a complementarity-determining region (hereinafter abbreviated as "CDR") into the framework regions (hereinafter abbreviated as "FR") of the above antibodies. The humanized antibody sc(Fv)<sub>2</sub> was expressed in CHO-DG44 cells, and its agonistic activity was assessed using BaF-human Mpl. The agonistic activity was used as an index to generate a humanized VB22B sc(Fv)<sub>2</sub> which has agonistic activity equivalent to that of murine VB22B sc(Fv)<sub>2</sub> by replacing one or more amino acids in its framework region.

[0197]

Specifically, synthetic oligo-DNAs of approximately 50 nucleotides in length were designed as to make 20 of these nucleotides available for hybridization, and the synthetic oligo-DNAs were assembled by PCR to prepare genes that encode the respective variable regions. Using the resulting genes, sc(Fv)<sub>2</sub> was similarly prepared by the method described in

Example 2.3. The respective DNAs were cloned into a pCXND3 expression vector to construct three kinds of expression vectors, pCXND3-hVB22B p-z sc(Fv)<sub>2</sub>, pCXND3-hVB22B g-e sc(Fv)<sub>2</sub>, and pCXND3-hVB22B e sc(Fv)<sub>2</sub>, to which the humanized VB22B sc(Fv)<sub>2</sub> is inserted. The nucleotide sequence and the amino acid sequence of hVB22B p-z sc(Fv)<sub>2</sub> in the plasmid are shown in SEQ ID NOs: 1 and 2. The nucleotide sequence and the amino acid sequence of hVB22B g-e sc(Fv)<sub>2</sub> are shown in SEQ ID NOs: 253 and 254. The nucleotide sequence and the amino acid sequence of hVB22B e sc(Fv)<sub>2</sub> are shown in SEQ ID NOs: 259 and 260. The nucleotide sequence and the amino acid sequence of murine VB22B sc(Fv)<sub>2</sub> are shown in SEQ ID NOs: 263 and 264. The plasmids were expressed in CHO-DG44 cells by the method described in Example 2.4 and the purification was done using the method described in Example 2.6. The TPO-like agonistic activities of the purified samples were similarly determined using the method described in Example 2.8. The TPO-like agonistic activities of the purified murine VB22B sc(Fv)<sub>2</sub>, and humanized hVB22B e sc(Fv)<sub>2</sub> and hVB22B g-e sc(Fv)<sub>2</sub> in BaF3-human Mpl were assessed. The results are shown in Figures 19. The humanized VB22B sc(Fv)<sub>2</sub> showed comparable agonistic activities, suggesting that the humanization has no influence on the activity.

[0198]

[Example 3] Preparation of anti-Mpl diabodies by the AGS method

Anti-Mpl diabodies having agonistic activity were prepared by an Autocrine Growth Selection (AGS) method (see, WO 03/91424).

[0199]

### 3.1 Construction of a retrovirus library

Spleens were isolated from MRL/lpr mice immunized with shMPL-Flag by the method described in Example 1.5, and homogenized in TRIZOL Reagent (Invitrogen) using a Dounce homogenizer. After chloroform addition, the homogenized sample was shaken vigorously, the aqueous phase was removed and total RNA was extracted by isopropanol precipitation. mRNA was purified using a PolyAtract System 1000 (Promega). Reverse transcription of 2.5 µg mRNA was carried out at 42°C for 50 min using the Superscript First strand synthesis system for RT-PCR (Invitrogen) and the included oligo-dT primers to prepare cDNA.

[0200]

The composition of the PCR reaction solution (250 µL) is shown below.

10x KOD Plus Buffer (Toyobo)	25 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo)	25 µL
2.5 mM MgSO <sub>4</sub> (Toyobo)	10 µL
KOD Plus (Toyobo)	7.5 µL
Reverse transcription products	25 µL

Mixed primers complementary to H chain or L chain variable region 500 pmol  
[0201]

The reaction conditions were:

98°C (initial temperature) for 3 min;

32 cycles of 98°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec;

and final extension was at 72°C for 6 min.

[0202]

The H chain primer mix contained HS1 to HS19 (SEQ ID NOs: 178 to 196) and HA1 to HA4 (SEQ ID NOs: 197 to 200), which were mixed at the indicated ratios next to the sequence names in Table 1. The L chain primer mix contained LS1 to LS17 (SEQ ID NOs: 201 to 217), LSlambda (SEQ ID NO: 218), LA1 to LA5 (SEQ ID NOs: 219 to 222), and LAlambda (SEQ ID NO: 223). The respective PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The H chain and L chain variable regions were linked *via* the (Gly<sub>4</sub>Ser)<sub>1</sub> linker sequence by PCR using sc-S (SEQ ID NO: 224) and sc-AS (SEQ ID NO: 225) as described below.

[0203]

The composition of the PCR reaction solution (100 µL in total) is shown below.

10x KOD Plus Buffer (Toyobo)	10 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo)	10 µL
2.5 mM MgSO <sub>4</sub> (Toyobo)	4 µL
KOD Plus (Toyobo)	2 µL
Fragment of H chain variable region	4 µL
Fragment of L chain variable region	4 µL

[0204]

The first-round PCR conditions were:

94°C (initial temperature) for 3 min; and

seven cycles of 94°C for 1 min and 63°C for 4 min.

Then, sc-S and sc-AS (25 pmol each) were added to the first-round products.

The second-round PCR conditions were:

30 cycles of 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min;

and final extension was at 72°C for 6 min.

[0205]

The resulting product with an *Sfi*I restriction site at both ends was purified using the QIAquick PCR Purification Kit (QIAGEN), and incubated with the *Sfi*I restriction enzyme (TaKaRa) overnight at 50°C. The PCR product purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) was inserted into the *Sfi*I site of the viral vector

pMX/IL3ssGFPHis.

[0206]

The resulting plasmid was constructed by inserting a GFP gene, which has an *EcoRI* site, mouse IL-3 signal sequence and *SfiI* site at its 5' end; and an *SfiI* site, His tag sequence, 5 termination codon, and *NotI* site at its 3' end, between the *EcoRI* and *NotI* sites on the pMX viral vector (Onishi, M. *et al.*, Mol. Cell. Biol. 18: 3871-3879). The plasmid was introduced into the ElectroMAX DH10B T1 phage resistant cells (Invitrogen) by electroporation (settings: 2.5 kV, 25  $\mu$ F, and 100 $\Omega$ ) using a Gene Pulser II (Bio-Rad). The cells were plated onto an LB-Agar 10 plate containing 100  $\mu$ g/mL ampicillin. After overnight incubation,  $1 \times 10^7$  colonies were obtained. Colonies were recovered from the plate and plasmids were then extracted using the QIAGEN Plasmid Maxi Kit (QIAGEN).

[0207]



[Table 1]

SEQ ID NO:178 (HS1(4))	GCCCAGCCGGCCATGGCGGAKGTRMAGCTTCAGGAGTC
SEQ ID NO:179 (HS2(4))	GCCCAGCCGGCCATGGCGGAGGTBCAGCTBCAGCAGTC
SEQ ID NO:180 (HS3(3))	GCCCAGCCGGCCATGGCGCAGGTGCAGCTGAAGSASTC
SEQ ID NO:181 (HS4(4))	GCCCAGCCGGCCATGGCGGAGGTCCARCTGCAACARTC
SEQ ID NO:182 (HS5(7))	GCCCAGCCGGCCATGGCGCAGGTYCAGCTBCAGCARTC
SEQ ID NO:183 (HS6(2))	GCCCAGCCGGCCATGGCGCAGGTYCARCTGCAGCAGTC
SEQ ID NO:184 (HS7(1))	GCCCAGCCGGCCATGGCGCAGGTCCACGTGAAGCAGTC
SEQ ID NO:185 (HS8(2))	GCCCAGCCGGCCATGGCGGAGGTGAASSTGGTGGAAATC
SEQ ID NO:186 (HS9(5))	GCCCAGCCGGCCATGGCGGAVGTGAWGYTGGTGGAGTC
SEQ ID NO:187 (HS10(2))	GCCCAGCCGGCCATGGCGGAGGTGCAGSKGGTGGAGTC
SEQ ID NO:188 (HS11(2))	GCCCAGCCGGCCATGGCGGAKGTGCAMCTGGTGGAGTC
SEQ ID NO:189 (HS12(2))	GCCCAGCCGGCCATGGCGGAGGTGAAGCTGATGGARTC
SEQ ID NO:190 (HS13(1))	GCCCAGCCGGCCATGGCGGAGGTGCARCTTGTTGAGTC
SEQ ID NO:191 (HS14(2))	GCCCAGCCGGCCATGGCGGARGTRAAGCTTCTCGAGTC
SEQ ID NO:192 (HS15(2))	GCCCAGCCGGCCATGGCGGAAGTGAARSTTGAGGAGTC
SEQ ID NO:193 (HS16(5))	GCCCAGCCGGCCATGGCGCAGGTTACTCTRAAAGWGTSTG
SEQ ID NO:194 (HS17(3.5))	GCCCAGCCGGCCATGGCGCAGGTCCAAC TVCAGCARCC
SEQ ID NO:195 (HS18(0.7))	GCCCAGCCGGCCATGGCGGATGTGAACTTGGAAGTGTC
SEQ ID NO:196 (HS19(0.7))	GCCCAGCCGGCCATGGCGGAGGTGAAGGTCATCGAGTC
SEQ ID NO:197 (HA1(1))	GGAGCCGCCGCCGCCCGAGGAAACGGTGACCGTGGT
SEQ ID NO:198 (HA2(1))	GGAGCCGCCGCCGCCCGAGGAGACTGTGAGAGTGGT
SEQ ID NO:199 (HA3(1))	GGAGCCGCCGCCGCCCGCAGAGACAGTGACCAAGT
SEQ ID NO:200 (HA4(1))	GGAGCCGCCGCCGCCCGAGGAGACGGTGACTGAGGT
SEQ ID NO:201 (LS1(1))	GGCGGCGGCGGCTCCGAYATCCAGCTGACTCAGCC
SEQ ID NO:202 (LS2(2))	GGCGGCGGCGGCTCCGAYATTGTTCTCWCCAGTC
SEQ ID NO:203 (LS3(5))	GGCGGCGGCGGCTCCGAYATTGTGMTMACTCAGTC
SEQ ID NO:204 (LS4(3.5))	GGCGGCGGCGGCTCCGAYATTGTGYTRACACAGTC
SEQ ID NO:205 (LS5(4))	GGCGGCGGCGGCTCCGAYATTGTRATGACMCAGTC
SEQ ID NO:206 (LS6(7))	GGCGGCGGCGGCTCCGAYATTMAGATRAMCCAGTC
SEQ ID NO:207 (LS7(6))	GGCGGCGGCGGCTCCGAYATTCAGATGAYDCAGTC
SEQ ID NO:208 (LS8(1.5))	GGCGGCGGCGGCTCCGAYATYCAGATGACACAGAC
SEQ ID NO:209 (LS9(2))	GGCGGCGGCGGCTCCGAYATTGTTCTCAWCCAGTC
SEQ ID NO:210 (LS10(3.5))	GGCGGCGGCGGCTCCGAYATTGWGCTSACCCAATC
SEQ ID NO:211 (LS11(8))	GGCGGCGGCGGCTCCGAYATTSTRATGACCCARTC
SEQ ID NO:212 (LS12(8))	GGCGGCGGCGGCTCCGAYRTTKTGATGACCCARAC
SEQ ID NO:213 (LS13(6))	GGCGGCGGCGGCTCCGAYATTGTGATGACBCAGKC
SEQ ID NO:214 (LS14(2))	GGCGGCGGCGGCTCCGAYATTGTGATAACYCAGGA
SEQ ID NO:215 (LS15(2))	GGCGGCGGCGGCTCCGAYATTGTGATGACCCAGWT
SEQ ID NO:216 (LS16(1))	GGCGGCGGCGGCTCCGAYATTGTGATGACACAACC
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SEQ ID NO:218 (LS1lambda(1))	GGCGGCGGCGGCTCCGATGCTGTTGTGACTCAGGAATC
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SEQ ID NO:221 (LA4(4))	GGAATTCGGCCCCCGAGGCCTTTATTTCCAAC TTTG
SEQ ID NO:222 (LA5(4))	GGAATTCGGCCCCCGAGGCCTTCAGCTCCAGCTTGG
SEQ ID NO:223 (LA1lambda(1))	GGAATTCGGCCCCCGAGGCCCCCTAGGACAGTCAGTTTGG

[0208]

### 3.2 Establishment of autonomously replicating cell lines by the AGS method

The resulting library was transfected into a packaging cell, Pt-E, (Morita, S. *et al.*, Gene therapy 7: 1063-1066) using FuGENE 6 (Roche Diagnostics). Specifically, Pt-E was plated onto 6-cm dishes and cultured in DMEM/10% FBS (Invitrogen). A mixture of FuGENE 6 and the library was added to the plate the following day. The culture medium was exchanged the next day, and the culture supernatant was collected 24 hours after that. 10 µg/mL polybrene (Hexadimethrine Bromide; Sigma) and 2 ng/mL mIL-3 were added to the culture supernatant containing recombinant virus particles. The viral solution was used to infect the BaF-monkey Mpl target cells. The cells were washed with PBS the following day, and suspended in RPMI 1640/10% FBS without mIL-3. The suspension was plated onto a 96-well plate at a cell density of 1,000 cells/well. Autonomously replicating cell lines (AB317 and AB324) were obtained after seven days of incubation. Genomic DNAs were extracted from these cells using a DNeasy Tissue Kit (QIAGEN), and the antibody genes were amplified by PCR.

[0209]

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x LA Taq Buffer (TaKaRa)	5 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	5 µL
2.5 mM MgCl <sub>4</sub> (TaKaRa)	5 µL
TaKaRa LA Taq (TaKaRa)	0.5 µL
Genomic DNA	0.5 µg
AGSdbS1 (SEQ ID NO: 226) and AGSdbA1 (SEQ ID NO: 227)	25 pmol

[0210]

The reaction conditions were:

94°C (initial temperature) for 1 min;

30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 70°C for 1 min;

and final extension was at 72°C for 6 min.

[0211]

The nucleotide sequence and the amino acid sequence of the H chain of cloned AB317 are shown in SEQ ID NOs: 154 and 155. The nucleotide sequence and the amino acid sequence of AB317 L chain are shown in SEQ ID NOs: 156 and 157. The nucleotide sequence and the amino acid sequence of AB324 H chain are shown in SEQ ID NOs: 158 and 159. The nucleotide sequence and the amino acid sequence of AB324 L chain are shown in SEQ ID NOs: 160 and 161.

[0212]

### 3.3 Activity assays of the diabodies obtained by AGS method

Each of the anti-Mpl diabodies obtained above was inserted into the pCXND3 expression vector. The PCR primers used are a synthetic oligonucleotide complementary to the 5' end of the diabody and containing an *EcoRI* site, and a synthetic oligonucleotide complementary to the nucleotide sequence of the 3' end of the diabody and containing a FLAG tag and a *NotI* site. The PCR product thus obtained was inserted into pCXND3 between the *EcoRI* and *NotI* sites. The diabody was expressed transiently in COS7 cells by the method described in Example 2.4. The culture supernatant was removed and the activity of the diabody was evaluated.

[0213]

The binding activities of the diabodies were assessed by flow cytometry using CHO cells that express Mpl derived from various species (Fig. 8). AB317 was proven to bind to CHO-mouse Mpl.

[0214]

The TPO-like agonistic activities of the diabodies were evaluated using BaF-human Mpl, BaF-monkey Mpl, and BaF-mouse Mpl (Figures 9, 10, and 11). AB317 had the highest agonistic activity against human, monkey, and mouse Mpl, whereas AB324 showed the highest agonistic activity against human and monkey Mpl.

[0215]

This proves that anti-Mpl diabodies having high agonistic activity can be obtained by the AGS method.

[0216]

[Example 4] Agonistic activity assays of the anti-Mpl antibodies against mutant Mpl in congenital amegakaryocytic thrombocytopenia (CAMT) patients

4.1 Establishment of BaF3 cell lines introduced with the mutant Mpl observed in CAMT patients

Mutations on G305C (R102P), C769T (R257C), and C823A (P275T) have been reported in the Mpl gene of CAMT patients. The respective expression vectors carrying the Mpl gene mutations were constructed and introduced into BaF3 cells. The following Mpl gene fragments were constructed: normal Mpl gene (nucleotide sequence, SEQ ID NO: 246; amino acid sequence, SEQ ID NO: 123); gene G305C in which C is substituted for 305th nucleotide G relative to the initiation codon (nucleotide sequence, SEQ ID NO: 247; amino acid sequence, SEQ ID NO: 248); gene C769T in which T is substituted for 769th nucleotide C (nucleotide sequence, SEQ ID NO: 249; amino acid sequence, SEQ ID NO: 250); and gene C823A in which A is substituted for 823rd nucleotide C (nucleotide sequence, SEQ ID NO: 251; amino acid sequence, SEQ ID NO: 252). These DNA fragments were digested with *EcoRI* and *SalI*, and inserted between the *EcoRI* and *SalI* sites on the animal cell expression vector pCOS2-Ha to prepare pCOS2-hMPLfullG305C, pCOS2-hMPLfullC769T, and pCOS2-hMPLfullC823A.

[0217]

The genes were introduced into BaF3 cells by the procedure described in Example 1.1.1 to establish BaF3 cell lines expressing each Mpl gene: BaF3-human MPL (G305C), BaF3-human MPL (C769T), and BaF3-human MPL (C823A). After the selection, the cells  
5 were cultured and passaged using RPMI 1640 containing 1 ng/mL mIL-3 and 10% FBS.

[0218]

#### 4.2 Preparation of anti-human Mpl diabody and sc(Fv)<sub>2</sub>

Among the amino acid sequences shown in Figures 6 and 7, expression vectors were prepared for the diabodies VB8B, VB45B, VB33, VB140, VB157, and TA136 using the same  
10 procedure described in Example 2.2.2. The prepared expression vectors were introduced into COS7 cells by the same procedure described in Example 2.2.4. The supernatant concentration of each diabody was determined by the method of Example 2.2.5. The sc(Fv)<sub>2</sub> expression vector for TA136 was prepared by the same procedure described in Example 2.2.3. The vector was introduced into CHO-DG44 cells by the same procedure described in Example 2.2.4.

15 sc(Fv)<sub>2</sub> was purified from the culture supernatant thus obtained using the same method described in Example 2.2.6.

[0219]

#### 4.3 Agonistic activity assays of sc(Fv)<sub>2</sub> and the anti-human Mpl diabodies

The prepared diabodies and sc(Fv)<sub>2</sub> were assayed for their agonistic activities in normal  
20 Mpl and mutant Mpl in BaF3 cells by the same procedure described in Example 2.2.8. The agonistic activities in BaF3-human Mpl and BaF3-human Mpl (G305C) were compared using the culture supernatants of cells expressing the diabodies. The TA136 diabody (TA136 db) was shown to have a low agonistic activity in BaF3-human Mpl cells expressing the normal Mpl gene, and a high agonistic activity in BaF3-human Mpl (G305C) cells expressing the mutant Mpl gene.  
25 hTPO and the rest of the diabodies did not show a high agonistic activity in BaF3-human Mpl (G305C) cells (Figures 12 and 13).

[0220]

In addition, the agonistic activities of the TA136 diabody and TA136 sc(Fv)<sub>2</sub> in  
30 BaF3-human Mpl, BaF3-human Mpl (G305C), BaF3-human Mpl (C769T), and BaF3-human Mpl (C823A) cells were assessed using a purified sample of the diabody. Compared with hTPO and the TA136 diabody, TA136 sc(Fv)<sub>2</sub> exhibited a higher agonistic activity in all three types of the TPO receptor mutant cell lines (Figures 15, 16 and 17). Furthermore, it was shown that in BaF3-human Mpl cells expressing the normal Mpl gene, the TA136 diabody exhibited a lower activity than hTPO. However, an agonistic activity equivalent to that of hTPO was  
35 achieved by converting the diabody into sc(Fv)<sub>2</sub> (Fig. 14).



[Brief Description of the Drawings]

[0221]

[Fig. 1] Fig. 1 demonstrates the strategy for preparing single-chain antibody  $sc(Fv)_2$ .

[Fig. 2] Fig. 2 illustrates the assessment of VB22B  $sc(Fv)_2$  binding activity using an

5 Mpl-expressing CHO cell line. Purified VB22B  $sc(Fv)_2$  was used.

[Fig. 3] Fig. 3 illustrates the assessment of VB22B antibody agonistic activity using BaF-human Mpl.

[Fig. 4] Fig. 4 illustrates the assessment of VB22B antibody agonistic activity using BaF-monkey Mpl.

10 [Fig. 5] Fig. 5 illustrates the assessment of VB22B antibody agonistic activity using M-07e.

[Fig. 6] Fig. 6 shows the amino acid sequences of anti-human Mpl antibodies (H chains) that exhibit higher agonistic activities when converted into minibodies.

15 [Fig. 7] Fig. 7 shows the amino acid sequences of anti-human Mpl antibodies (L chains) which exhibit higher agonistic activities when converted into minibodies.

[Fig. 8] Fig. 8 illustrates the binding activity assessment of AB317 diabody using Mpl-expressing CHO cells. Both VB22B diabody (solid line) and AB317 diabody (broken line) were obtained from COS7 culture supernatants.

20 [Fig. 9] Fig. 9 illustrates the agnostic activity assessment of AB324 and AB317 diabolies using BaF-human Mpl.

[Fig. 10] Fig. 10 illustrates the agnostic activity assessment of AB324 and AB317 diabolies using BaF-monkey Mpl.

[Fig. 11] Fig. 11 illustrates the agnostic activity assessment of AB324 and AB317 diabolies using BaF-mouse Mpl.

25 [Fig. 12] Fig. 12 shows the agonistic activities of diabolies and hTPO in BaF3-human Mpl cells. The Y-axis shows OD at 450/655 nm, and the X-axis represents concentration.

[Fig.13] Fig. 13 shows the agonistic activities of diabolies and hTPO in BaF3-human Mpl (G305C) cells. The Y-axis shows OD at 450/655 nm, and the X-axis represents concentration.

30 [Fig. 14] Fig. 14 shows the agonistic activities of TA136 db and TA136  $sc(Fv)_2$  in BaF3-human Mpl cells. The Y-axis shows OD at 450/655 nm and the X-axis represents concentration.

[Fig. 15] Fig. 15 shows the agonistic activities of TA136 db and TA136  $sc(Fv)_2$  in BaF3-human Mpl (G305C) cells. The Y-axis shows OD at 450/655 nm, and the X-axis  
35 represents concentration.

[Fig. 16] Fig. 16 shows the agonistic activities of TA136 db and TA136  $sc(Fv)_2$  in



BaF3-human Mpl (C769T) cells. The Y-axis shows OD at 450/655 nm, and the X-axis represents concentration.

[Fig. 17] Fig. 17 shows the agonistic activities of TA136 db and TA136 sc(Fv)<sub>2</sub> in BaF3-human Mpl (C823A) cells. The Y-axis shows OD at 450/655 nm, and the X-axis represents concentration.

[Fig. 18] Fig. 18 shows the positions of FRs and CDRs in humanized heavy chain sequences (hVB22B p-z: VH), and humanized light chain sequences (hVB22B p-z: VL).

[Fig. 19] Fig. 19 shows the TPO-like agonistic activities of murine VB22B sc(Fv)<sub>2</sub>, hVB22B e sc(Fv)<sub>2</sub>, and hVB22B g-e sc(Fv)<sub>2</sub> in BaF3-human Mpl. The Y-axis shows absorbance ratio (450nm/655nm), and the X-axis represents concentration.

## [Sequence Listing]

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Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val  
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Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe

85 90 95

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Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser  
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5 Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu  
 515 520 525

Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg  
 530 535 540

10 Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys  
 545 550 555 560

Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu  
 15 565 570 575

Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met Asp Tyr Arg  
 580 585 590

20 Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro  
 595 600 605

Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile Ala Asn His  
 610 615 620

25 Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro  
 625 630 635

30 <210> 124  
 <211> 122  
 <212> PRT  
 <213> Mus musculus

35 <400> 124  
 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala

1 5 10 15  
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser  
20 25 30  
5  
Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45  
Gly Arg Thr Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe  
10 50 55 60  
Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr  
65 70 75 80  
15 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95  
Ala Arg Gly Trp Ile Leu Ala Asp Gly Gly Tyr Ser Phe Ala Tyr Trp  
100 105 110  
20  
Gly Gln Gly Thr Leu Val Thr Val Ser Ala  
115 120  
25 <210> 125  
<211> 112  
<212> PRT  
<213> Mus musculus  
30 <400> 125  
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro Val Thr Pro Gly  
1 5 10 15  
Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
35 20 25 30



Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
5 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

10 Ser Arg Val Glu Ala Glu Asp Val Gly Ile Tyr Tyr Cys Met Gln His  
85 90 95

Leu Glu Tyr Pro Phe Thr Phe Gly Thr Gly Thr Lys Leu Glu Ile Lys  
100 105 110

15

<210> 126  
<211> 118  
<212> PRT  
20 <213> Mus musculus

<400> 126  
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

25 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser  
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
30 35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe  
50 55 60

35 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr  
65 70 75 80

Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

5 Ala Arg Gly Tyr Ala Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

Leu Val Thr Val Ser Ala  
115

10

<210> 127  
<211> 112  
<212> PRT  
15 <213> Mus musculus

<400> 127  
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

20 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
25 35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

30 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

35 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys

100

105

110

&lt;210&gt; 128

5 &lt;211&gt; 118

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 128

10 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser  
20 25 30

15 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe  
20 50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Asn Thr Ala Tyr  
65 70 75 80

25 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

Ala Arg Gly Phe Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

30 Leu Val Thr Val Ser Ala  
115

35 &lt;210&gt; 129

&lt;211&gt; 112

<212> PRT

<213> Mus musculus

<400> 129

5 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15  
Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30  
10 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45  
Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Ala Pro  
15 50 55 60  
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80  
20 Ser Arg Val Glu Thr Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95  
Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

25

<210> 130

<211> 118

<212> PRT

30 <213> Mus musculus

<400> 130

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15  
35 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Ser Ser

20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

5

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe  
50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr  
10 65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

15 Ala Ser Gly Tyr Ala Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

Leu Val Thr Val Ser Ala  
115

20

<210> 131  
<211> 112  
<212> PRT  
25 <213> Mus musculus

<400> 131  
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

30

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
5 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

10 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

<210> 132  
15 <211> 118  
<212> PRT  
<213> Mus musculus

<400> 132  
20 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Arg Ser  
20 25 30

25 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe  
30 50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr  
65 70 75 80

35 Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

Ala Ser Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

5 Leu Val Thr Val Ser Ala  
115

<210> 133

10 <211> 112

<212> PRT

<213> Mus musculus

<400> 133

15 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

20 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

25 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

30 Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

35



<210> 134

<211> 118

<212> PRT

<213> Mus musculus

5

<400> 134

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

10 Ser Val Lys Ile Ser Cys Arg Ala Phe Gly Tyr Ala Phe Ser Asn Ser  
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

15

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe  
50 55 60

20 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr  
65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

25 Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

Leu Val Thr Val Ser Ala  
115

30

<210> 135

<211> 112

<212> PRT

35 <213> Mus musculus

&lt;400&gt; 135

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

5 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

10

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

15 Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

20 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

&lt;210&gt; 136

25 &lt;211&gt; 115

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 136

30 Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr  
20 25 30

35

Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile

35 40 45

Gly Arg Ile His Pro Ser Asp Ser Glu Thr His Cys Asn Gln Lys Phe  
50 55 60

5

Lys Arg Lys Ala Thr Leu Thr Val Asn Lys Ser Ser Ser Thr Ala Tyr  
65 70 75 80

Ile Gln Leu His Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys  
10 85 90 95

Thr Ser Gly Gly Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr  
100 105 110

15 Val Ser Ala  
115

<210> 137

20 <211> 112  
<212> PRT  
<213> Mus musculus

<400> 137

25 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser  
20 25 30

30 Asn Gly Asn Ile Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
35 50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
5 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

10  
<210> 138  
<211> 118  
<212> PRT  
<213> Mus musculus

15  
<400> 138  
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

20 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser  
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

25  
Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe  
50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Thr Thr Ala Tyr  
30 65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

35 Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

Leu Val Thr Val Ser Ala

115

5

<210> 139

<211> 112

<212> PRT

<213> Mus musculus

10

<400> 139

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly

1 5 10 15

15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser

20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser

35 40 45

20

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro

50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile

25 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His

85 90 95

30

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys

100 105 110

<210> 140

35

<211> 118

<212> PRT

<213> Mus musculus

<400> 140

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
5 1 5 10 15  
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Thr Ser  
20 25 30  
10 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45  
Gly Arg Ile Tyr Pro Gly Asp Gly Glu Ala Asn Tyr Asn Gly Lys Phe  
50 55 60  
15 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ser Ala Tyr  
65 70 75 80  
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
20 85 90 95  
Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110  
25 Leu Val Thr Val Ser Ala  
115

<210> 141

30 <211> 112

<212> PRT

<213> Mus musculus

<400> 141

35 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

5 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Met Gln Arg Pro Gly Gln Ser  
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

10 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
15 85 90 95

Val Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

20

<210> 142  
<211> 118  
<212> PRT  
<213> Mus musculus

25

<400> 142  
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

30 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser  
20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Pro Glu Trp Ile  
35 40 45

35 Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe



50 55 60

Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr  
65 70 75 80

5

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
10 100 105 110

Leu Val Thr Val Ser Ala  
115

15

<210> 143  
<211> 112  
<212> PRT  
<213> Mus musculus

20

<400> 143  
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

25

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

30

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
35 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
5 100 105 110

<210> 144  
<211> 118  
10 <212> PRT  
<213> Mus musculus

<400> 144  
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Leu Asn Pro Gly Ala  
15 1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Arg Ser  
20 20 25 30

Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Tyr Asn Gly Lys Phe  
50 55 60

25 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Thr Thr Ala Tyr  
65 70 75 80

Met Gln Phe Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
30 85 90 95

Ala Arg Gly Asp Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

35 Leu Val Thr Val Ser Ala  
115

<210> 145

<211> 112

5 <212> PRT

<213> Mus musculus

<400> 145

10 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

15 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

20 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
25 85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

30

<210> 146

<211> 115

<212> PRT

<213> Mus musculus

35

<400> 146

Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly Ala  
 1 5 10 15  
 Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr  
 5 20 25 30  
 Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile  
 35 40 45  
 10 Gly Arg Ile His Pro Tyr Asp Ser Glu Thr His Tyr Asn Gln Lys Phe  
 50 55 60  
 Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr  
 65 70 75 80  
 15 Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys  
 85 90 95  
 Ala Ser Gly Gly Trp Phe Ala Ser Trp Gly Gln Gly Thr Leu Val Thr  
 20 100 105 110  
 Val Ser Ala  
 115  
 25  
 <210> 147  
 <211> 112  
 <212> PRT  
 <213> Mus musculus  
 30  
 <400> 147  
 Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
 1 5 10 15  
 35 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser  
 20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

5 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Thr Ile  
65 70 75 80

10 Ser Ser Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
15 100 105 110

<210> 148  
<211> 115  
20 <212> PRT  
<213> Mus musculus

<400> 148  
Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala  
25 1 5 10 15

Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr  
20 25 30

30 Trp Met Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile  
35 40 45

Gly Arg Ile His Pro Phe Asp Ser Glu Thr His Cys Ser Gln Lys Phe  
50 55 60

35 Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Asn Thr Ala Tyr

30

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

5 <210> 150  
<211> 118  
<212> PRT  
<213> Mus musculus

10 <400> 150  
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

15 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Ser  
20 25 30

Trp Met Asn Trp Val Arg Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
35 40 45

20 Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn Gly Lys Phe  
50 55 60

Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr  
65 70 75 80

25 Met Glu Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

30 Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

Leu Val Thr Val Ser Ala  
115

35 <210> 151



<211> 112

<212> PRT

<213> Mus musculus

5 <400> 151

Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly  
1 5 10 15

10 Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Asn  
20 25 30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

15 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
50 55 60

20 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
85 90 95

25 Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
100 105 110

<210> 152

<211> 118

30 <212> PRT

<213> Mus musculus

<400> 152

35 Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala  
1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Ser Asn Ser  
20 25 30

Trp Met Asn Trp Val Asn Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile  
5 35 40 45

Gly Arg Ile Tyr Pro Gly Asp Gly Asp Thr Ile Tyr Asn Gly Asn Phe  
50 55 60

10 Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Ile Ala Tyr  
65 70 75 80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys  
85 90 95

15 Thr Ser Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr  
100 105 110

Leu Val Thr Val Ser Ala  
20 115

<210> 153  
<211> 112  
25 <212> PRT  
<213> Mus musculus

<400> 153  
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Leu Pro Val Thr Pro Gly  
30 1 5 10 15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser  
20 25 30

35 Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser  
35 40 45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
 50 55 60

5 Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile  
 65 70 75 80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His  
 85 90 95

10 Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys  
 100 105 110

15 <210> 154  
 <211> 423  
 <212> DNA  
 <213> Mus musculus

20 <220>  
 <221> CDS  
 <222> (1).. (423)  
 <223>

25 <400> 154  
 atg gtt ctt gcc agc tct acc acc agc atc cac acc atg ctg ctc ctg 48  
 Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu  
 1 5 10 15

30 ctc ctg atg ctg gcc cag ccg gcc atg gcg gaa gtg aag ctg gtg gag 96  
 Leu Leu Met Leu Ala Gln Pro Ala Met Ala Glu Val Lys Leu Val Glu  
 20 25 30

tct ggg gga ggc tta gtg aag cct gga ggg tcc cgg aaa ctc tcc tgt 144  
 35 Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Arg Lys Leu Ser Cys  
 35 40 45

gca gcc tct gga ttc act ttc agt agc tat acc atg tct tgg gtt cgc 192  
Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Thr Met Ser Trp Val Arg  
50 55 60

5  
cag act ccg gcg aag agg ctg gag tgg gtc gca acc att agt agt ggc 240  
Gln Thr Pro Ala Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly  
65 70 75 80

10  
agt agt acc atc tac tat gca gac aca gtg aag ggc cga ttc acc atc 288  
Ser Ser Thr Ile Tyr Tyr Ala Asp Thr Val Lys Gly Arg Phe Thr Ile  
85 90 95

15  
tcc aga gac aat gcc aag aac acc ctg ttc ctg caa atg acc agt cta 336  
Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu  
100 105 110

20  
agg tct gag gac aca gcc atg tat tac tgt gca agg aga tgg ttt ctt 384  
Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Trp Phe Leu  
115 120 125

25  
gac tgc tgg ggc caa ggc acc act ctc aca gtc tcc tcg 423  
Asp Cys Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser  
130 135 140

30  
<210> 155  
<211> 141  
<212> PRT  
<213> Mus musculus

35  
<400> 155  
Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu  
1 5 10 15  
Leu Leu Met Leu Ala Gln Pro Ala Met Ala Glu Val Lys Leu Val Glu

20 25 30

Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser Arg Lys Leu Ser Cys  
35 40 45

5  
Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Thr Met Ser Trp Val Arg  
50 55 60

Gln Thr Pro Ala Lys Arg Leu Glu Trp Val Ala Thr Ile Ser Ser Gly  
10 65 70 75 80

Ser Ser Thr Ile Tyr Tyr Ala Asp Thr Val Lys Gly Arg Phe Thr Ile  
85 90 95

15 Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu  
100 105 110

Arg Ser Glu Asp Thr Ala Met Tyr Tyr Cys Ala Arg Arg Trp Phe Leu  
115 120 125

20  
Asp Cys Trp Gly Gln Gly Thr Thr Leu Thr Val Ser Ser  
130 135 140

25 <210> 156  
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30 <220>  
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<222> (1).. (357)  
<223>

35 <400> 156  
gat att gtg ctc acc caa tct cca gct tct ttg gct gtg tct cta ggg

	Asp	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Ser	Leu	Ala	Val	Ser	Leu	Gly	
	1				5					10					15		
	cag agt gtc acc atc tcc tgc aga gcc agt gaa agt gtt gaa tat tat																96
5	Gln	Ser	Val	Thr	Ile	Ser	Cys	Arg	Ala	Ser	Glu	Ser	Val	Glu	Tyr	Tyr	
				20					25					30			
	ggc act agt tta atg cag tgg tac caa cag aaa cca gga cag cca ccc																144
	Gly	Thr	Ser	Leu	Met	Gln	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Gln	Pro	Pro	
10				35				40						45			
	aaa ctc ctc atc tat ggt gca tcc aac gta gaa tct ggg gtc cct gcc																192
	Lys	Leu	Leu	Ile	Tyr	Gly	Ala	Ser	Asn	Val	Glu	Ser	Gly	Val	Pro	Ala	
			50				55					60					
15																	
	agg ttt agt ggc agt ggg tct ggg aca gac ttc agc ctc aac atc cat																240
	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Ser	Leu	Asn	Ile	His	
	65					70				75					80		
20	cct gtg gag gag gat gat att gca atg tat ttc tgt cag caa agt agg																288
	Pro	Val	Glu	Glu	Asp	Asp	Ile	Ala	Met	Tyr	Phe	Cys	Gln	Gln	Ser	Arg	
					85					90					95		
	aag gtt ccg tgg acg ttc ggt gga ggc acc aag ctg gaa ata aag gac																336
25	Lys	Val	Pro	Trp	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Asp	
				100					105					110			
	tac aag gat gac gac gat aag																357
	Tyr	Lys	Asp	Asp	Asp	Asp	Lys										
30				115													
	<210> 157																
	<211> 119																
35	<212> PRT																
	<213> Mus musculus																

<400> 157

Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly

1 5 10 15

5

Gln Ser Val Thr Ile Ser Cys Arg Ala Ser Glu Ser Val Glu Tyr Tyr

20 25 30

Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro

10 35 40 45

Lys Leu Leu Ile Tyr Gly Ala Ser Asn Val Glu Ser Gly Val Pro Ala

50 55 60

15 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Ser Leu Asn Ile His

65 70 75 80

Pro Val Glu Glu Asp Asp Ile Ala Met Tyr Phe Cys Gln Gln Ser Arg

85 90 95

20

Lys Val Pro Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Asp

100 105 110

Tyr Lys Asp Asp Asp Asp Lys

25 115

<210> 158

<211> 432

30 <212> DNA

<213> Mus musculus

<220>

<221> CDS

35 <222> (1)..(432)

<223>



&lt;400&gt; 158

	atg gtt ctt gcc agc tct acc acc agc atc cac acc atg ctg ctc ctg	48
	Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu	
5	1                      5                      10                      15	
	ctc ctg atg ctg gcc cag ccg gcc atg gcg cag gtt cag ctc cag caa	96
	Leu Leu Met Leu Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln	
	20                      25                      30	
10	tct gga cct gag ctg gtg aag cct ggg gcc tca gtg aag att tcc tgc	144
	Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys	
	35                      40                      45	
15	aag gct tct ggc tat gca ttc agt agc tcc tgg atg aac tgg atg aag	192
	Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser Trp Met Asn Trp Met Lys	
	50                      55                      60	
	cag agg cct gga aag ggt ctt gag tgg att ggg cgg att tat cct gga	240
20	Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile Gly Arg Ile Tyr Pro Gly	
	65                      70                      75                      80	
	gat gga gat act aac tac aat ggg aag ttc aag ggc aag gcc aca ctg	288
	Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu	
25	85                      90                      95	
	act gca gac aaa tcc tcc agc aca gcc tac atg caa ctc agc agc ctg	336
	Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu	
	100                      105                      110	
30	aca tct gag gac tct gcg gtc tac ttc tgt gca aga gcg agg aaa act	384
	Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Ala Arg Lys Thr	
	115                      120                      125	
35	tcc tgg ttt gct tac tgg ggc caa ggg act ctg gtc act gtc tct gcg	432
	Ser Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala	

130

135

140

&lt;210&gt; 159

5 &lt;211&gt; 144

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 159

10 Met Val Leu Ala Ser Ser Thr Thr Ser Ile His Thr Met Leu Leu Leu  
1 5 10 15

Leu Leu Met Leu Ala Gln Pro Ala Met Ala Gln Val Gln Leu Gln Gln  
20 25 30

15 Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys  
35 40 45

Lys Ala Ser Gly Tyr Ala Phe Ser Ser Ser Trp Met Asn Trp Met Lys  
20 50 55 60

Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile Gly Arg Ile Tyr Pro Gly  
65 70 75 80

25 Asp Gly Asp Thr Asn Tyr Asn Gly Lys Phe Lys Gly Lys Ala Thr Leu  
85 90 95

Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met Gln Leu Ser Ser Leu  
100 105 110

30 Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys Ala Arg Ala Arg Lys Thr  
115 120 125

Ser Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ala  
35 130 135 140

<210> 160  
 <211> 345  
 <212> DNA  
 5 <213> Mus musculus  
  
 <220>  
 <221> CDS  
 <222> (1).. (345)  
 10 <223>  
  
 <400> 160  
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 Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly  
 15 1 5 10 15  
  
 gac agg gtc agc atc agc tgc aag gcc agt cag aat gtg ggt aat att 96  
 Asp Arg Val Ser Ile Ser Cys Lys Ala Ser Gln Asn Val Gly Asn Ile  
 20 20 25 30  
 20  
 ata gcc tgg tat caa cag aaa cca ggg caa tct cct aaa gca ctg att 144  
 Ile Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile  
 35 40 45  
 25 tac ttg gca tcc tac cgg tac agt gga gtc cct gat cgc ttc aca ggc 192  
 Tyr Leu Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly  
 50 55 60  
 agt gga tct ggg aca gat ttc act ctc acc att agt aat gtg cag tct 240  
 30 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser  
 65 70 75 80  
 gaa gac ttg gca gag tat ttc tgt cag caa tat agc agc tct ccg ctc 288  
 Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Pro Leu  
 35 85 90 95

acg ttc ggt gct ggg acc aag ctg gaa ata aag gac tac aag gat gac 336  
Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp  
100 105 110

5 gac gat aag 345  
Asp Asp Lys  
115

10 <210> 161  
<211> 115  
<212> PRT  
<213> Mus musculus

15 <400> 161  
Asp Ile Val Leu Thr Gln Ser Gln Lys Phe Met Ser Thr Ser Val Gly  
1 5 10 15  
Asp Arg Val Ser Ile Ser Cys Lys Ala Ser Gln Asn Val Gly Asn Ile  
20 20 25 30  
Ile Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile  
35 40 45  
Tyr Leu Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly  
50 55 60  
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser  
65 70 75 80  
30 Glu Asp Leu Ala Glu Tyr Phe Cys Gln Gln Tyr Ser Ser Ser Pro Leu  
85 90 95  
Thr Phe Gly Ala Gly Thr Lys Leu Glu Ile Lys Asp Tyr Lys Asp Asp  
35 100 105 110

Asp Asp Lys

115

5 <210> 162

<211> 116

<212> PRT

<213> Mus musculus

10 <400> 162

Asp Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln

1 5 10 15

Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile Thr Ser Asp

15 20 25 30

Tyr Ala Trp Ser Trp Ile Arg Gln Leu Pro Gly Asn Lys Leu Glu Trp

35 40 45

20 Met Gly Tyr Ile Thr Tyr Ser Gly Tyr Ser Ile Tyr Asn Pro Ser Leu

50 55 60

Lys Ser Arg Ile Ser Ile Ser Arg Asp Thr Ser Lys Asn Gln Leu Phe

65 70 75 80

25

Leu Gln Leu Asn Ser Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys

85 90 95

Val Gly Gly Tyr Asp Asn Met Asp Tyr Trp Gly Gln Gly Thr Ser Val

30 100 105 110

Thr Val Ser Ser

115

35

<210> 163

<211> 108

<212> PRT

<213> *Mus musculus*

5 <400> 163

Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly  
1 5 10 15

10 Glu Lys Val Thr Leu Thr Cys Ser Ala Ser Ser Ser Val Ser Ser Ser  
20 25 30

His Leu Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys Leu Trp  
35 40 45

15 Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser  
50 55 60

Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Asn Met Glu  
65 70 75 80

20 Thr Glu Asp Ala Ala Ser Tyr Phe Cys His Gln Trp Ser Ser Tyr Pro  
85 90 95

25 Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys  
100 105

<210> 164

<211> 1924

30 <212> DNA

<213> *Macaca fascicularis*

<220>

<221> CDS

35 <222> (11).. (1918)

<223>

&lt;400&gt; 164

	gaattccacc atg ccc tcc tgg gcc ctc ttc atg gtc acc tcc tgc ctc	49
	Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu	
5	1 5 10	
	ctc ctg gcc cct caa aac ctg gcc caa gtc agc agc caa gat gtc tcc	97
	Leu Leu Ala Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser	
	15 20 25	
10		
	ttg ctg gcc tcg gac tca gag ccc ctg aag tgt ttc tcc cga aca ttt	145
	Leu Leu Ala Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe	
	30 35 40 45	
15		
	gag gac ctc act tgc ttc tgg gat gag gaa gag gca gca ccc agt ggg	193
	Glu Asp Leu Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly	
	50 55 60	
	aca tac cag ctg ctg tat gcc tac ccg ggg gag aag ccc cgt gcc tgc	241
20	Thr Tyr Gln Leu Leu Tyr Ala Tyr Pro Gly Glu Lys Pro Arg Ala Cys	
	65 70 75	
	ccc ctg agt tct cag agc gtg ccc cgc ttt gga acc cga tac gtg tgc	289
	Pro Leu Ser Ser Gln Ser Val Pro Arg Phe Gly Thr Arg Tyr Val Cys	
25	80 85 90	
	cag ttt cca gcc cag gaa gaa gtg cgt ctc ttc tct ccg ctg cac ctc	337
	Gln Phe Pro Ala Gln Glu Glu Val Arg Leu Phe Ser Pro Leu His Leu	
	95 100 105	
30		
	tgg gtg aag aat gtg ttc cta aac cag act cag att cag cga gtc ctc	385
	Trp Val Lys Asn Val Phe Leu Asn Gln Thr Gln Ile Gln Arg Val Leu	
	110 115 120 125	
35		
	ttt gtg gac agt gta ggc ctg ccg gct ccc ccc agt atc atc aag gcc	433
	Phe Val Asp Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala	



	130	135	140	
	atg ggt ggg agc cag cca ggg gaa ctt cag atc agc tgg gag gcc cca			481
	Met Gly Gly Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro			
5	145	150	155	
	gct cca gaa atc agt gat ttc ctg agg tac gaa ctc cgc tat ggc ccc			529
	Ala Pro Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro			
	160	165	170	
10				
	aaa gat ctc aag aac tcc act ggt ccc acg gtc ata cag ttg atc gcc			577
	Lys Asp Leu Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala			
	175	180	185	
15	aca gaa acc tgc tgc cct gct ctg cag agg cca cac tca gcc tct gct			625
	Thr Glu Thr Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala			
	190	195	200	205
	ctg gac cag tct cca tgt gct cag ccc aca atg ccc tgg caa gat gga			673
20	Leu Asp Gln Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly			
	210	215	220	
	cca aag cag acc tcc cca act aga gaa gct tca gct ctg aca gca gtg			721
	Pro Lys Gln Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val			
25	225	230	235	
	ggt gga agc tgc ctc atc tca gga ctc cag cct ggc aac tcc tac tgg			769
	Gly Gly Ser Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp			
	240	245	250	
30				
	ctg cag ctg cgc agc gaa cct gat ggg atc tcc ctc ggt ggc tcc tgg			817
	Leu Gln Leu Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp			
	255	260	265	
35	gga tcc tgg tcc ctc cct gtg act gtg gac ctg cct gga gat gca gtg			865
	Gly Ser Trp Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val			

	270	275	280	285	
	gca att gga ctg caa tgc ttt acc ttg gac ctg aag aat gtt acc tgt				913
	Ala Ile Gly Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys				
5		290	295	300	
	caa tgg cag caa gag gac cat gct agt tcc caa ggt ttc ttc tac cac				961
	Gln Trp Gln Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His				
		305	310	315	
10					
	agc agg gca cgg tgc tgc ccc aga gac agg tac ccc atc tgg gag gac				1009
	Ser Arg Ala Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp				
		320	325	330	
15					
	tgt gaa gag gaa gag aaa aca aat cca gga tta cag acc cca cag ttc				1057
	Cys Glu Glu Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe				
		335	340	345	
	tct cgc tgc cac ttc aag tca cga aat gac agc gtt att cac atc ctt				1105
20	Ser Arg Cys His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu				
		350	355	360	365
	gtg gag gtg acc aca gcc ctg ggt gct gtt cac agt tac ctg ggc tcc				1153
	Val Glu Val Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser				
25		370	375	380	
	cct ttc tgg atc cac cag gct gtg cgc ctc ccc acc cca aac ttg cac				1201
	Pro Phe Trp Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His				
		385	390	395	
30					
	tgg agg gag atc tcc agc ggg cat ctg gaa ttg gag tgg cag cac cca				1249
	Trp Arg Glu Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro				
		400	405	410	
35					
	tca tcc tgg gca gcc caa gag acc tgc tat caa ctc cga tac aca gga				1297
	Ser Ser Trp Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly				

	415	420	425													
	gaa ggc cat cag gac tgg aag gtg ctg gag ccg cct ctc ggg gcc cga															1345
	Glu Gly His Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg															
5	430	435	440	445												
	gga ggg acc ctg gag ctg cgc ccg cga tct cgc tac cgt tta cag ctg															1393
	Gly Gly Thr Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu															
	450					455					460					
10																
	cgc gcc agg ctc aat ggc ccc acc tac caa ggt ccc tgg agc tcg tgg															1441
	Arg Ala Arg Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp															
	465					470					475					
15																
	tcg gac cca gct agg gtg gag acc gcc acc gag acc gcc tgg att tcc															1489
	Ser Asp Pro Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser															
	480					485					490					
	ttg gtg acc gct ctg ctg cta gtg ctg ggc ctc agc gcc gtc ctg ggc															1537
20	Leu Val Thr Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly															
	495					500					505					
	ctg ctg ctg ctg agg tgg cag ttt cct gca cac tac agg aga ctg agg															1585
	Leu Leu Leu Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg															
25	510	515	520	525												
	cat gcc ctg tgg ccc tca ctt cca gat ctg cac cga gtc cta ggc cag															1633
	His Ala Leu Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln															
	530					535					540					
30																
	tac ctt agg gac act gca gcc ctg agt ccg ccc aag gcc aca gtc tca															1681
	Tyr Leu Arg Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser															
	545					550					555					
35	gat acc tgt gaa gaa gtg gaa ccc agc ctc ctt gaa atc ctc ccc aag															1729
	Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys															

560 565 570

tcc tca gag agg act cct ttg ccc ctg tgt tcc tcc cag tcc cag atg 1777  
Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met  
5 575 580 585

gac tac cga aga ttg cag cct tct tgc ctg ggg acc atg ccc ctg tct 1825  
Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser  
590 595 600 605

10  
gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att 1873  
Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile  
610 615 620

15 gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga 1918  
Ala Asn His Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro  
625 630 635

20  
gtcgac 1924

<210> 165  
<211> 635  
<212> PRT  
25 <213> Macaca fascicularis

<400> 165  
Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu Leu Leu Ala  
1 5 10 15

30  
Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser Leu Leu Ala  
20 25 30

Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu  
35 35 40 45

Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln  
50 55 60

Leu Leu Tyr Ala Tyr Pro Gly Glu Lys Pro Arg Ala Cys Pro Leu Ser  
5 65 70 75 80

Ser Gln Ser Val Pro Arg Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro  
85 90 95

10 Ala Gln Glu Glu Val Arg Leu Phe Ser Pro Leu His Leu Trp Val Lys  
100 105 110

Asn Val Phe Leu Asn Gln Thr Gln Ile Gln Arg Val Leu Phe Val Asp  
115 120 125

15 Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly  
130 135 140

Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro Ala Pro Glu  
20 145 150 155 160

Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Lys Asp Leu  
165 170 175

25 Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr  
180 185 190

Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln  
195 200 205

30 Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln  
210 215 220

Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val Gly Gly Ser  
35 225 230 235 240

Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu  
245 250 255

5 Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp  
260 265 270

Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Ile Gly  
275 280 285

10 Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln  
290 295 300

Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala  
305 310 315 320

15 Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp Cys Glu Glu  
325 330 335

Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys  
20 340 345 350

His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu Val Glu Val  
355 360 365

25 Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser Pro Phe Trp  
370 375 380

Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu  
385 390 395 400

30 Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp  
405 410 415

Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His  
35 420 425 430

Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr  
435 440 445

5 Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg  
450 455 460

Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro  
465 470 475 480

10 Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr  
485 490 495

Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu  
500 505 510

15 Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu  
515 520 525

Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg  
20 530 535 540

Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys  
545 550 555 560

25 Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu  
565 570 575

Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met Asp Tyr Arg  
580 585 590

30 Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro  
595 600 605

Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile Ala Asn His  
35 610 615 620



Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro  
625 630 635

5	<210>	166
	<211>	24
	<212>	DNA
	<213>	Artificial

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10    <220>
      <223>  an artificially synthesized sequence
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<400> 166  
caggggccag tggatagact gatg

24

15

<210>	167
<211>	23
<212>	DNA
<213>	Artificial

20

<220>  
<223> an artificially synthesized sequence

25 <400> 167  
gctcactgga tgggtgggaag atg

23

<210>	168
<211>	30
<212>	DNA
<213>	Artificial

30

<220>  
<223> an artificially synthesized primer sequence

35

<400> 168

tagaattcca ccatggaatg gcctttgatc

30

5 <210> 169

<211> 56

<212> DNA

<213> Artificial

10 <220>

<223> an artificially synthesized primer sequence

<400> 169

agcctgagtc atcacaatat ccgatccgcc tccacctgca gagacagtga ccagag

56

15

<210> 170

<211> 56

<212> DNA

20 <213> Artificial

<220>

<223> an artificially synthesized primer sequence

25 <400> 170

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56

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 Glu Asp Leu Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly  
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 Thr Tyr Gln Leu Leu Tyr Ala Tyr Pro Arg Glu Lys Pro Arg Ala Cys  
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	Gln Phe Pro Asp Gln Glu Glu Val Pro Leu Phe Phe Pro Leu His Leu	
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	Trp Val Lys Asn Val Phe Leu Asn Gln Thr Arg Thr Gln Arg Val Leu	
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	Phe Val Asp Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala	
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	Ala Pro Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro	
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	Arg Asp Pro Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala	
	175 180 185	
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	Leu Asp Gln Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly	
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	cca aag cag acc tcc cca agt aga gaa gct tca gct ctg aca gca gag	721
35	Pro Lys Gln Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu	
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	Leu Gln Leu Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp	
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	Gly Ser Trp Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val	
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	gca ctt gga ctg caa tgc ttt acc ttg gac ctg aag aat gtt acc tgt	913
15	Ala Leu Gly Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys	
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	caa tgg cag caa cag gac cat gct agc tcc caa ggc ttc ttc tac cac	961
	Gln Trp Gln Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His	
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	agc agg gca cgg tgc tgc ccc aga gac agg tac ccc atc tgg gag aac	1009
	Ser Arg Ala Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn	
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25	tgc gaa gag gaa gag aaa aca aat cca gga cta cag acc cca cag ttc	1057
	Cys Glu Glu Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe	
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30	tct cgc tgc cac ttc aag tca cga aat gac agc att att cac atc ctt	1105
	Ser Arg Cys His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu	
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	Pro Phe Trp Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His	
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	Trp Arg Glu Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro	
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	Ser Ser Trp Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly	
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	Gly Gly Thr Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu	
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	cgc gcc agg ctc aac ggc ccc acc tac caa ggt ccc tgg agc tcg tgg	1441
	Arg Ala Arg Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp	
	465 470 475	
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	Ser Asp Pro Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser	
	480 485 490	
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	Leu Val Thr Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly	
	495 500 505	
	ctg ctg ctg ctg agg tgg cag ttt cct gca cac tac agg aga ctg agg	1585
35	Leu Leu Leu Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg	
	510 515 520 525	

cat gcc ctg tgg ccc tca ctt cca gac ctg cac cgg gtc cta ggc cag 1633  
His Ala Leu Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln  
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Tyr Leu Arg Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser  
545 550 555

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gat acc tgt gaa gaa gtg gaa ccc agc ctc ctt gaa atc ctc ccc aag 1729  
Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys  
560 565 570

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Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met  
575 580 585

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gac tac cga aga ttg cag cct tct tgc ctg ggg acc atg ccc ctg tct 1825  
Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser  
590 595 600 605

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gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att 1873  
Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile  
610 615 620

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gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga 1918  
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	Pro	Gln	Asn	Leu	Ala	Gln	Val	Ser	Ser	Gln	Asp	Val	Ser	Leu	Leu	Ala
				20					25					30		
	Ser	Asp	Ser	Glu	Pro	Leu	Lys	Cys	Phe	Ser	Arg	Thr	Phe	Glu	Asp	Leu
10			35					40					45			
	Thr	Cys	Phe	Trp	Asp	Glu	Glu	Glu	Ala	Ala	Pro	Ser	Gly	Thr	Tyr	Gln
		50					55					60				
15	Leu	Leu	Tyr	Ala	Tyr	Pro	Arg	Glu	Lys	Pro	Arg	Ala	Cys	Pro	Leu	Ser
	65					70					75					80
	Ser	Gln	Ser	Met	Pro	His	Phe	Gly	Thr	Arg	Tyr	Val	Cys	Gln	Phe	Pro
					85					90					95	
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	Asp	Gln	Glu	Glu	Val	Pro	Leu	Phe	Phe	Pro	Leu	His	Leu	Trp	Val	Lys
					100					105					110	
	Asn	Val	Phe	Leu	Asn	Gln	Thr	Arg	Thr	Gln	Arg	Val	Leu	Phe	Val	Asp
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	Ser	Val	Gly	Leu	Pro	Ala	Pro	Pro	Ser	Ile	Ile	Lys	Ala	Met	Gly	Gly
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	145					150					155					160
	Ile	Ser	Asp	Phe	Leu	Arg	Tyr	Glu	Leu	Arg	Tyr	Gly	Pro	Arg	Asp	Pro
					165					170					175	
35																
	Lys	Asn	Ser	Thr	Gly	Pro	Thr	Val	Ile	Gln	Leu	Ile	Ala	Thr	Glu	Thr

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	Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu Gly Gly Ser		
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	Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu		
	245	250	255
15	Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp		
	260	265	270
	Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Leu Gly		
	275	280	285
20	Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln		
	290	295	300
	Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala		
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	Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn Cys Glu Glu		
	325	330	335
30	Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys		
	340	345	350
	His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu Val Glu Val		
	355	360	365
35	Thr Thr Ala Pro Gly Thr Val His Ser Tyr Leu Gly Ser Pro Phe Trp		

	370	375	380
	Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu		
	385	390	395 400
5	Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp		
	405	410	415
	Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His		
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	Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr		
	435	440	445
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	450	455	460
	Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro		
	465	470	475 480
20	Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr		
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	Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu		
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	Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu		
	515	520	525
30	Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg		
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	Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys		
	545	550	555 560
35	Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu		

565 570 575

Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met Asp Tyr Arg  
580 585 590

5 Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro  
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Leu Leu Ala Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser  
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	aca tac cag ctg ctg tat gcc tac ccg cgg gag aag ccc cgt gct tgc																241
	Thr Tyr Gln Leu Leu Tyr Ala Tyr Pro Arg Glu Lys Pro Arg Ala Cys																
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	Pro Leu Ser Ser Gln Ser Met Pro His Phe Gly Thr Arg Tyr Val Cys																
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	Gln Phe Pro Asp Gln Glu Glu Val Arg Leu Phe Phe Pro Leu His Leu																
		95				100				105							
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20	Trp Val Lys Asn Val Phe Leu Asn Gln Thr Arg Thr Gln Arg Val Leu																
	110				115				120					125			
	ttt gtg gac agt gta ggc ctg ccg gct ccc ccc agt atc atc aag gcc																433
	Phe Val Asp Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala																
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	Met Gly Gly Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Glu Pro																
			145				150					155					
30	gct cca gaa atc agt gat ttc ctg agg tac gaa ctc cgc tat ggc ccc																529
	Ala Pro Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro																
		160				165				170							
35	aga gat ccc aag aac tcc act ggt ccc acg gtc ata cag ctg att gcc																577
	Arg Asp Pro Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala																



	175	180	185	
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	Thr Glu Thr Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala			
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	ctg gac cag tct cca tgt gct cag ccc aca atg ccc tgg caa gat gga			673
	Leu Asp Gln Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly			
		210	215	220
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	cca aag cag acc tcc cca agt aga gaa gct tca gct ctg aca gca gag			721
	Pro Lys Gln Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu			
		225	230	235
15				
	ggt gga agc tgc ctc atc tca gga ctc cag cct ggc aac tcc tac tgg			769
	Gly Gly Ser Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp			
		240	245	250
	ctg cag ctg tgc agc gaa cct gat ggg atc tcc ctc ggt ggc tcc tgg			817
20	Leu Gln Leu Cys Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp			
		255	260	265
	gga tcc tgg tcc ctc cct gtg act gtg gac ctg cct gga gat gca gtg			865
	Gly Ser Trp Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val			
25	270	275	280	285
	gca ctt gga ctg caa tgc ttt acc ttg gac ctg aag aat gtt acc tgt			913
	Ala Leu Gly Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys			
		290	295	300
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	caa tgg cag caa cag gac cat gct agc tcc caa ggc ttc ttc tac cac			961
	Gln Trp Gln Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His			
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	agc agg gca cgg tgc tgc ccc aga gac agg tac ccc atc tgg gag aac			1009
	Ser Arg Ala Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn			

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	tgc gaa gag gaa gag aaa aca aat cca gga cta cag acc cca cag ttc	1057		
	Cys Glu Glu Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe			
5	335	340	345	
	tct cgc tgc cac ttc aag tca cga aat gac agc att att cac atc ctt	1105		
	Ser Arg Cys His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu			
	350	355	360	365
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	gtg gag gtg acc aca gcc ccg ggt act gtt cac agc tac ctg ggc tcc	1153		
	Val Glu Val Thr Thr Ala Pro Gly Thr Val His Ser Tyr Leu Gly Ser			
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	Pro Phe Trp Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His			
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	tgg agg gag atc tcc agt ggg cat ctg gaa ttg gag tgg cag cac cca	1249		
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	Ser Ser Trp Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly			
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	gaa ggc cat cag gac tgg aag gtg ctg gag ccg cct ctc ggg gcc cga	1345		
	Glu Gly His Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg			
	430	435	440	445
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	gga ggg acc ctg gag ctg cgc ccg cga tct cgc tac cgt tta cag ctg	1393		
	Gly Gly Thr Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu			
	450	455	460	
35	cgc gcc agg ctc aac ggc ccc acc tac caa ggt ccc tgg agc tcg tgg	1441		
	Arg Ala Arg Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp			

	465	470	475	
	tcg gac cca act agg gtg gag acc gcc acc gag acc gcc tgg atc tcc			1489
	Ser Asp Pro Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser			
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	ttg gtg acc gct ctg cat cta gtg ctg ggc ctc agc gcc gtc ctg ggc			1537
	Leu Val Thr Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly			
	495	500	505	
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	ctg ctg ctg ctg agg tgg cag ttt cct gca cac tac agg aga ctg agg			1585
	Leu Leu Leu Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg			
	510	515	520	525
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	cat gcc ctg tgg ccc tca ctt cca gac ctg cac cgg gtc cta ggc cag			1633
	His Ala Leu Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln			
	530	535	540	
	tac ctt agg gac act gca gcc ctg agc ccg ccc aag gcc aca gtc tca			1681
20	Tyr Leu Arg Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser			
	545	550	555	
	gat acc tgt gaa gaa gtg gaa ccc agc ctc ctt gaa atc ctc ccc aag			1729
	Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys			
25	560	565	570	
	tcc tca gag agg act cct ttg ccc ctg tgt tcc tcc cag gcc cag atg			1777
	Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met			
	575	580	585	
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	gac tac cga aga ttg cag cct tct tgc ctg ggg acc atg ccc ctg tct			1825
	Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser			
	590	595	600	605
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	gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att			1873
	Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile			

	610	615	620	
	gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga			1918
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	gtcgac			1924
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	Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu			
	35	40	45	
25	Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln			
	50	55	60	
	Leu Leu Tyr Ala Tyr Pro Arg Glu Lys Pro Arg Ala Cys Pro Leu Ser			
	65	70	75	80
30	Ser Gln Ser Met Pro His Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro			
	85	90	95	
	Asp Gln Glu Glu Val Arg Leu Phe Phe Pro Leu His Leu Trp Val Lys			
35	100	105	110	

Asn Val Phe Leu Asn Gln Thr Arg Thr Gln Arg Val Leu Phe Val Asp  
115 120 125

5 Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly  
130 135 140

Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Glu Pro Ala Pro Glu  
145 150 155 160

10 Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Arg Asp Pro  
165 170 175

Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr  
180 185 190

15 Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln  
195 200 205

Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln  
20 210 215 220

Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu Gly Gly Ser  
225 230 235 240

25 Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu  
245 250 255

Cys Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp  
260 265 270

30 Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Leu Gly  
275 280 285

Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln  
35 290 295 300

Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala  
305 310 315 320

Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn Cys Glu Glu  
5 325 330 335

Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys  
340 345 350

His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu Val Glu Val  
10 355 360 365

Thr Thr Ala Pro Gly Thr Val His Ser Tyr Leu Gly Ser Pro Phe Trp  
370 375 380

Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu  
15 385 390 395 400

Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp  
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Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His  
420 425 430

Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr  
25 435 440 445

Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg  
450 455 460

Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro  
30 465 470 475 480

Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr  
35 485 490 495

Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu  
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Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu  
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Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg  
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10 Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys  
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Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu  
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15 Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met Asp Tyr Arg  
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Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro  
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	Leu Leu Ala Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser	
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	110 115 120 125	
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	Arg Asp Pro Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala	
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	Ser	Arg	Cys	His	Phe	Lys	Ser	Arg	Asn	Asp	Ser	Ile	Ile	His	Ile	Leu	
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	Trp	Arg	Glu	Ile	Ser	Ser	Gly	His	Leu	Glu	Leu	Glu	Trp	Gln	His	Pro	
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	Arg	Ala	Arg	Leu	Asn	Gly	Pro	Thr	Tyr	Gln	Gly	Pro	Trp	Ser	Ser	Trp	
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	His	Ala	Leu	Trp	Pro	Ser	Leu	Pro	Asp	Leu	His	Arg	Val	Leu	Gly	Gln	
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Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys  
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5 Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met  
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10 Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser  
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gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att 1873  
Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile  
610 615 620

15 gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga 1918  
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35 Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu  
35 40 45

Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln  
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5 Leu Leu Tyr Ala Tyr Pro Arg Glu Lys Pro Arg Ala Cys Pro Leu Ser  
65 70 75 80

Ser Gln Ser Met Pro His Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro  
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10 Asp Gln Glu Glu Val Arg Leu Phe Phe Pro Leu His Leu Trp Val Lys  
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Asn Val Phe Leu Asn Gln Thr Arg Thr Gln Arg Val Leu Phe Val Asp  
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Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly  
130 135 140

20 Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Glu Pro Ala Pro Glu  
145 150 155 160

Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Arg Asp Pro  
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25 Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr  
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Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln  
30 195 200 205

Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln  
210 215 220

35 Thr Ser Pro Ser Arg Glu Ala Ser Ala Leu Thr Ala Glu Gly Gly Ser  
225 230 235 240

Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu  
245 250 255

5 Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp  
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Ser Leu Thr Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Leu Gly  
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10 Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln  
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Gln Gln Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala  
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Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asn Cys Glu Glu  
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20 Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys  
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His Phe Lys Ser Arg Asn Asp Ser Ile Ile His Ile Leu Val Glu Val  
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25 Thr Thr Ala Pro Gly Thr Val His Ser Tyr Leu Gly Ser Pro Phe Trp  
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35 Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His  
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Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr  
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5 Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg  
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Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro  
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10 Thr Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr  
485 490 495

Ala Leu His Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu  
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Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu  
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20 Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg  
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Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys  
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25 Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu  
565 570 575

Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ala Gln Met Asp Tyr Arg  
30 580 585 590

Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro  
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Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val  
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Tyr Tyr Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly  
115 120 125

25 Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly  
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165 170 175

Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr Trp  
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Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met  
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5 Ser Asn Leu Ala Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser  
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225 230 235 240

10 Gly Val Tyr Tyr Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly  
245 250 255

Gln Gly Thr Lys Leu Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly Gly  
260 265 270

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275 280 285

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25 Gly Lys Gly Leu Glu Trp Val Gly Arg Ile Tyr Pro Gly Asp Gly Glu  
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Thr Ile Tyr Asn Gly Lys Phe Arg Val Arg Val Thr Ile Thr Ala Asp  
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30 Glu Ser Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu  
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Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Val Met  
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420 425 430

10 Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr  
435 440 445

Tyr Leu Tyr Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu  
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15 Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro Asp Arg Phe Ser  
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Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Lys Ile Ser Arg Val Glu  
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5 aatgggaaat tcagggtcag agtcacgatt accgcggacg aatccacgag cacagcctac 240  
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30 Arg Val Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr  
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Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys  
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5 Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro  
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Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Lys Ile  
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Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe

20 35 40 45

Thr Asn Ser Trp Met Asn Trp Ile Arg Gln Arg Pro Gly Lys Gly Leu

50 55 60

25 Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn

65 70 75 80

Gly Lys Phe Arg Val Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser

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Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val

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Tyr Tyr Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly

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Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met  
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Gly Thr Ala Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val  
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Gly Val Tyr Tyr Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly  
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260 265 270

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30 Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser  
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Gly Tyr Thr Phe Thr Asn Ser Trp Met Asn Trp Ile Arg Gln Arg Pro  
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Gly Lys Gly Leu Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu  
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5 Thr Ile Tyr Asn Gly Lys Phe Arg Val Arg Val Thr Ile Thr Ala Asp  
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Glu Ser Thr Ser Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu  
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10 Asp Thr Ala Val Tyr Tyr Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe  
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Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Gly Gly Gly  
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Thr Gln Ser Ala Leu Ser Leu Pro Val Thr Pro Gly Glu Pro Ala Ser  
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Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser Asn Gly Asn Thr  
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25 Tyr Leu Tyr Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu  
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Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro Asp Arg Phe Ser  
465 470 475 480

30 Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Lys Ile Ser Arg Val Glu  
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Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His Ile Glu Tyr Pro  
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 aatgggaaat tcagggtcag agtcacgatt accgcggacg aatccacgag cacagcctac 240  
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	gggaaattca gggtaaggc cacactgact gcagacaaat cctccagcac agcctacatg	300		
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ggttcgggtg gtggtggttc ggggtggtggc ggatcggata ttgtgatgac tcaggctgca 480

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20 Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe  
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Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu  
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Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val  
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Pro Ser Ile Pro Val Thr Pro Gly Glu Ser Val Ser Ile Ser Cys Arg  
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Phe Leu Gln Arg Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met  
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Ser Asn Leu Ala Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser  
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Glu Leu Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser  
290 295 300

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Lys Ser Ser Ser Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu  
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Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile Ser Arg Val Glu  
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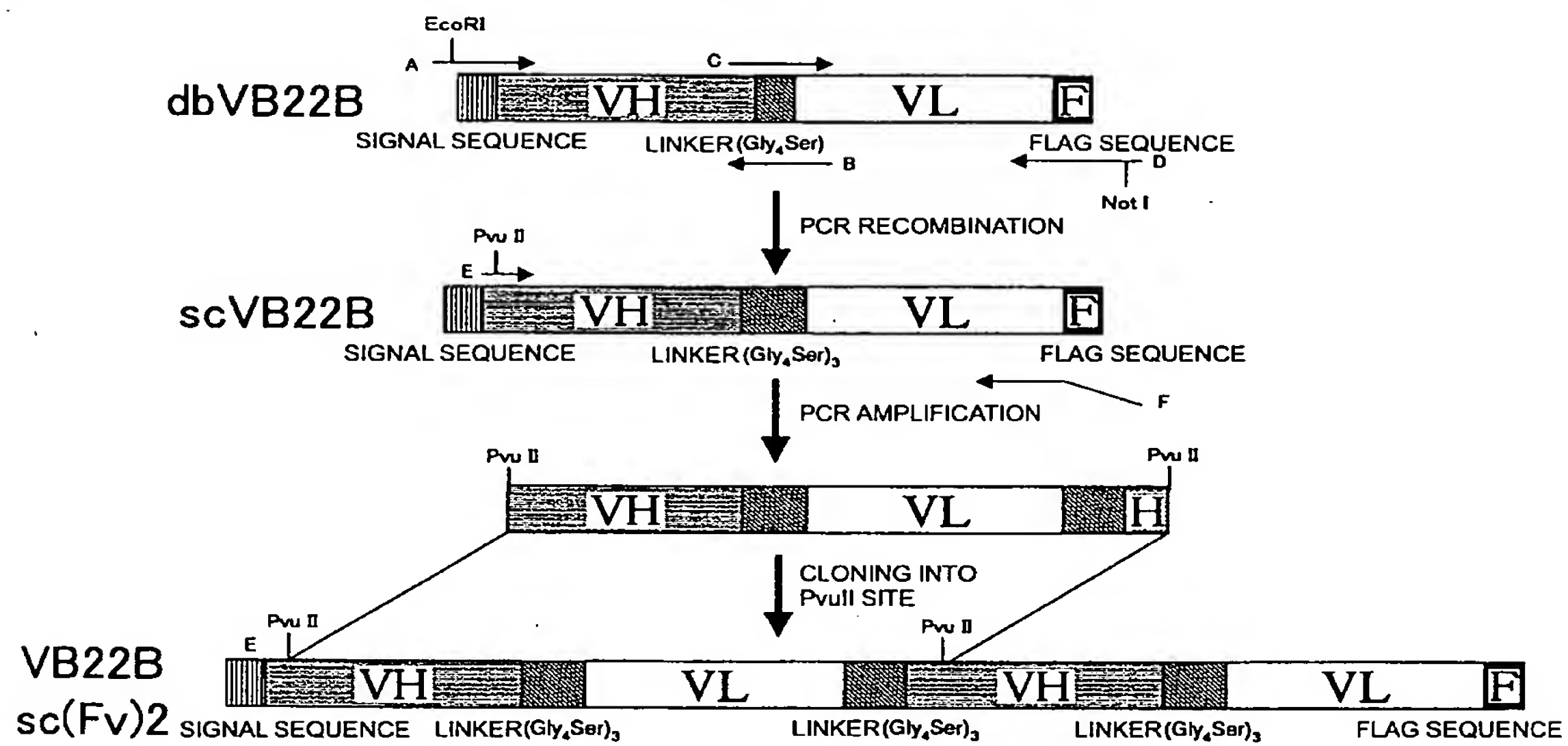
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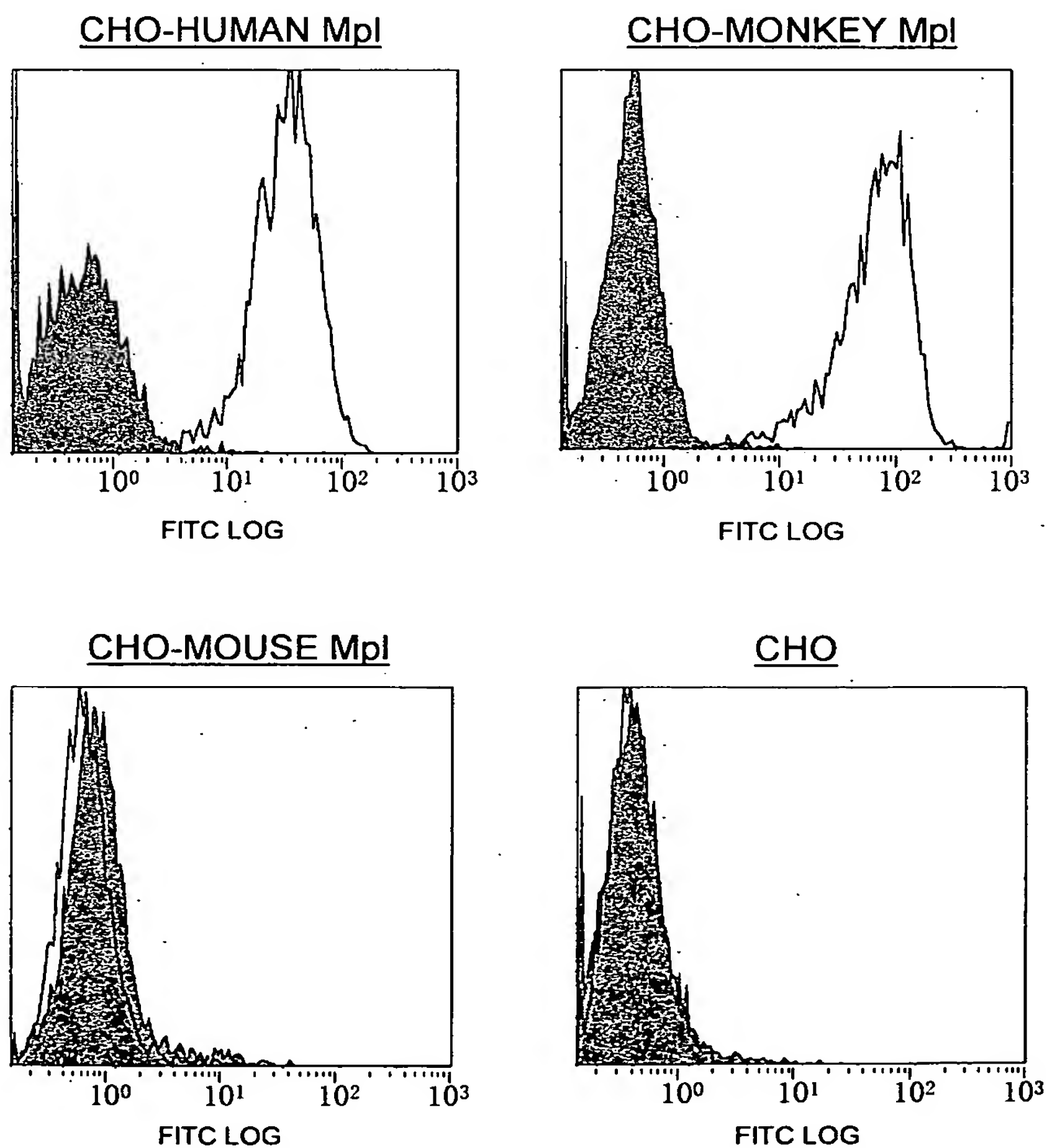


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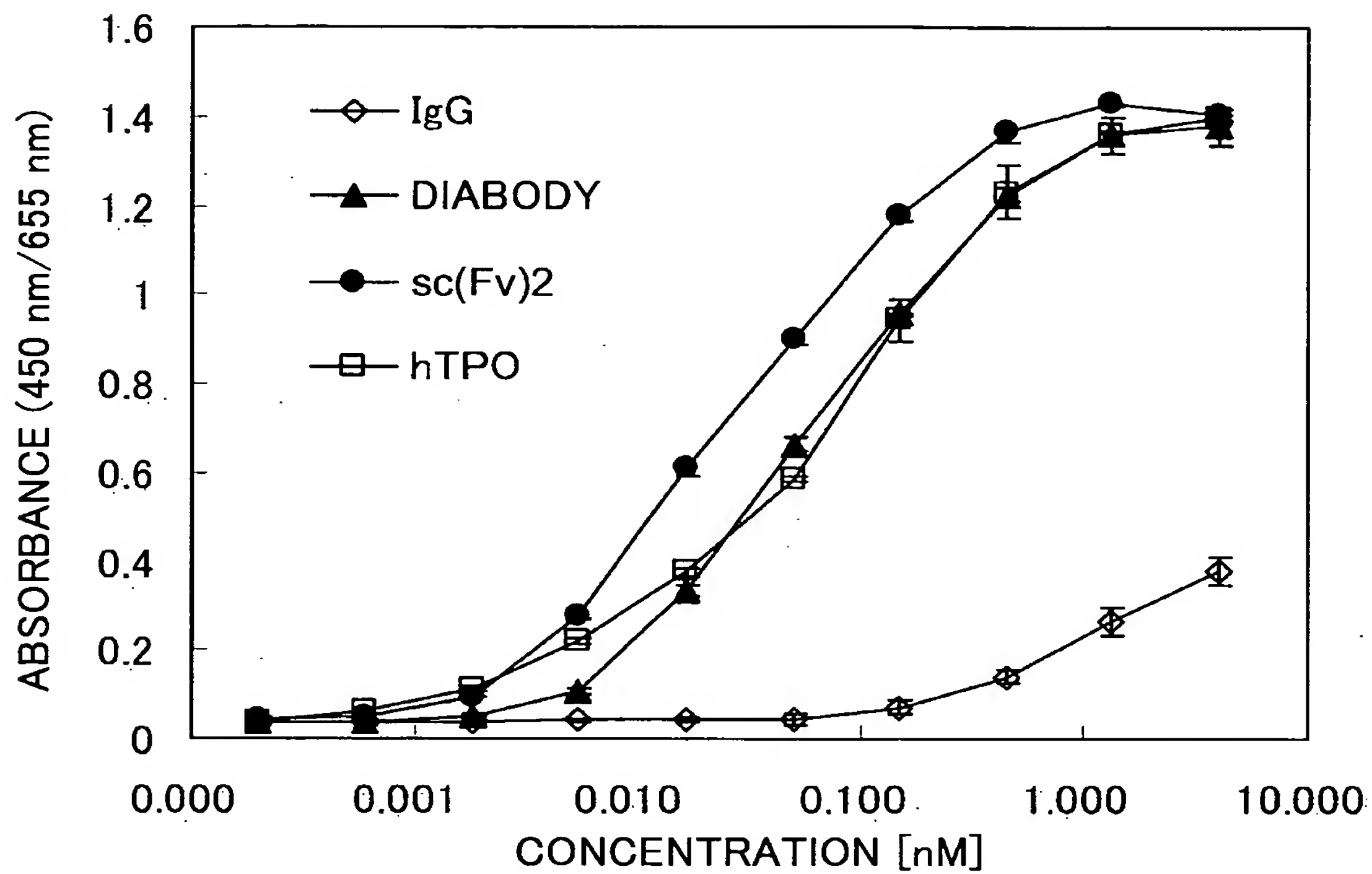
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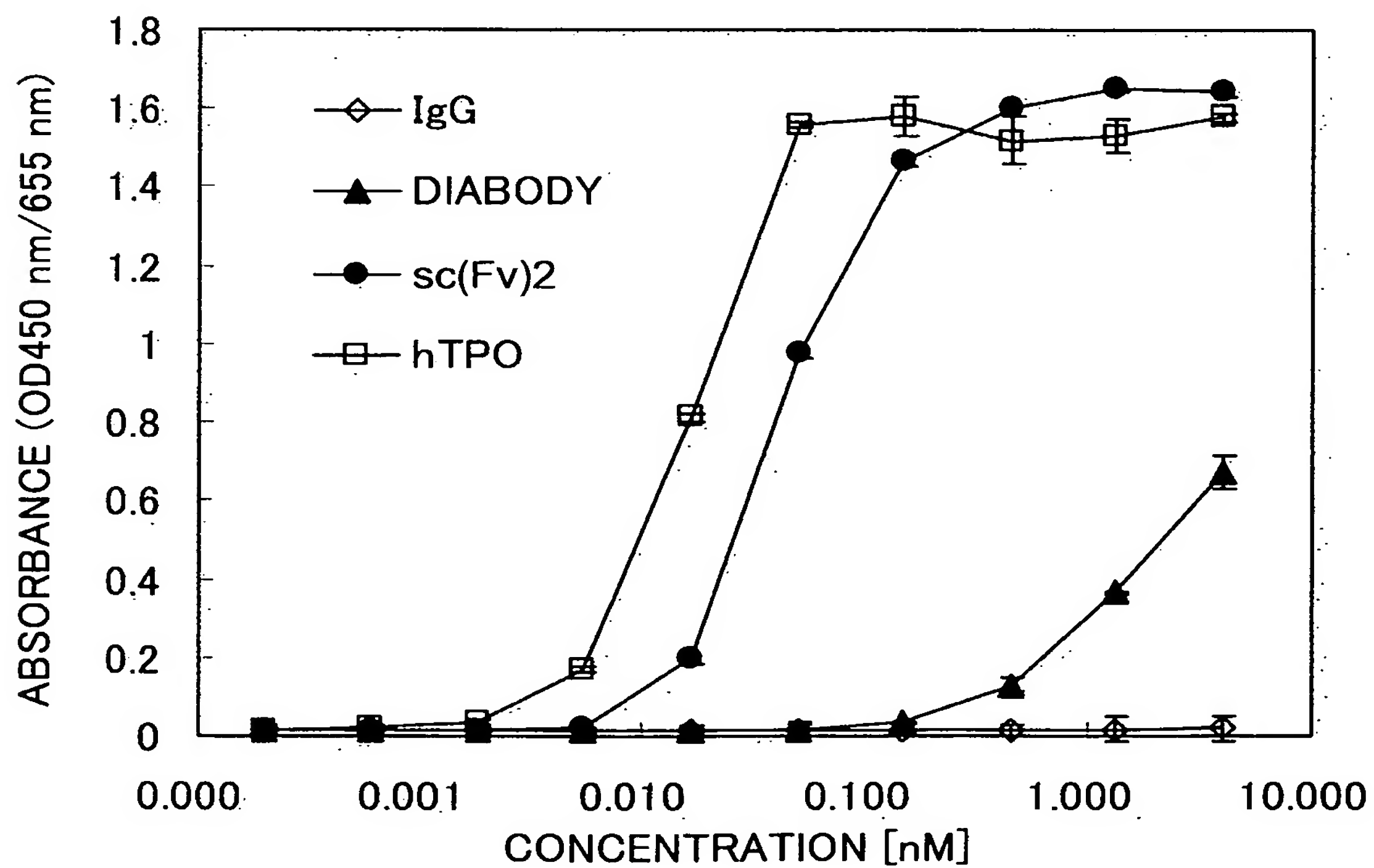
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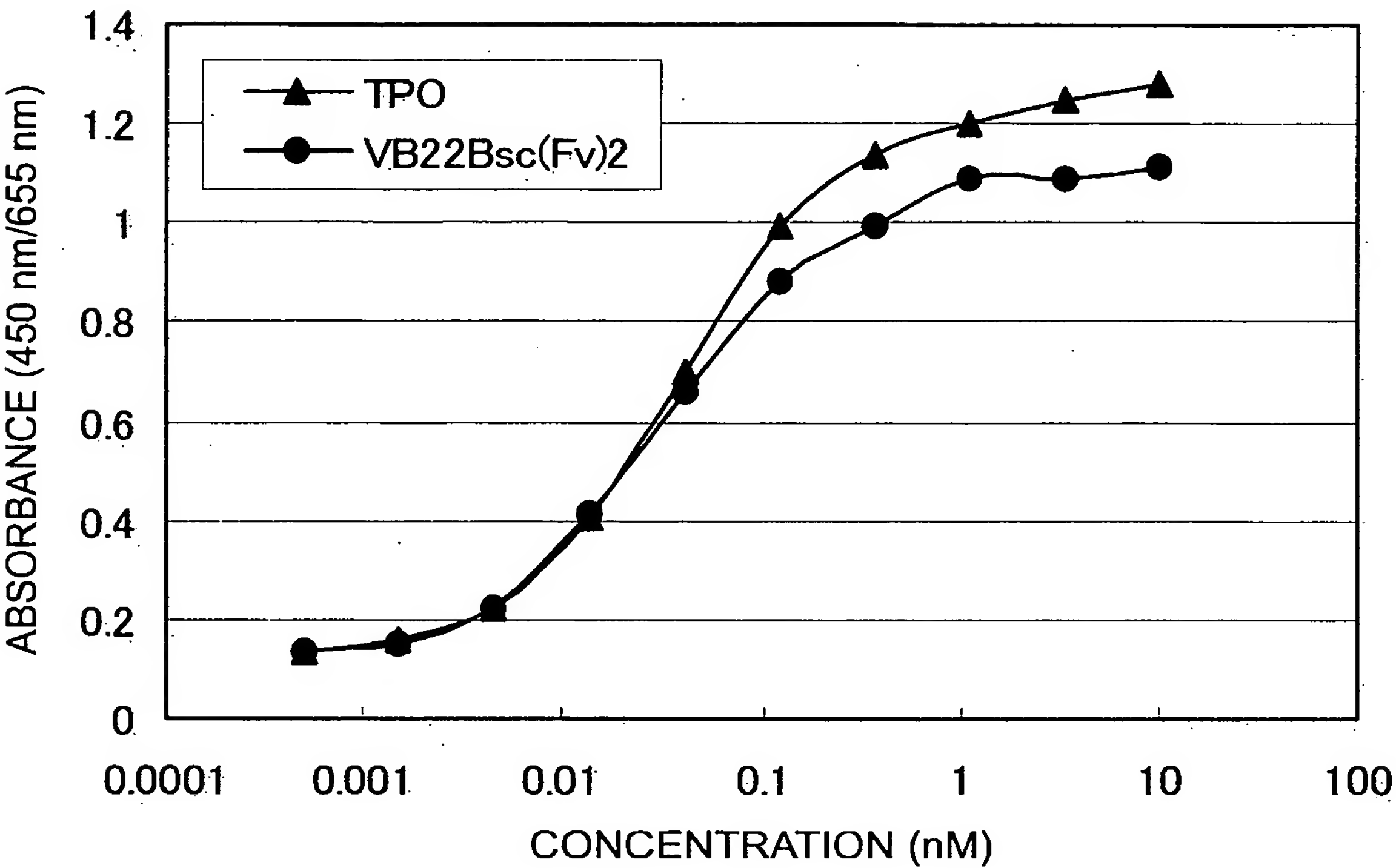
[Fig. 3]



[Fig.4]



[Fig. 5]



[Fig. 6]

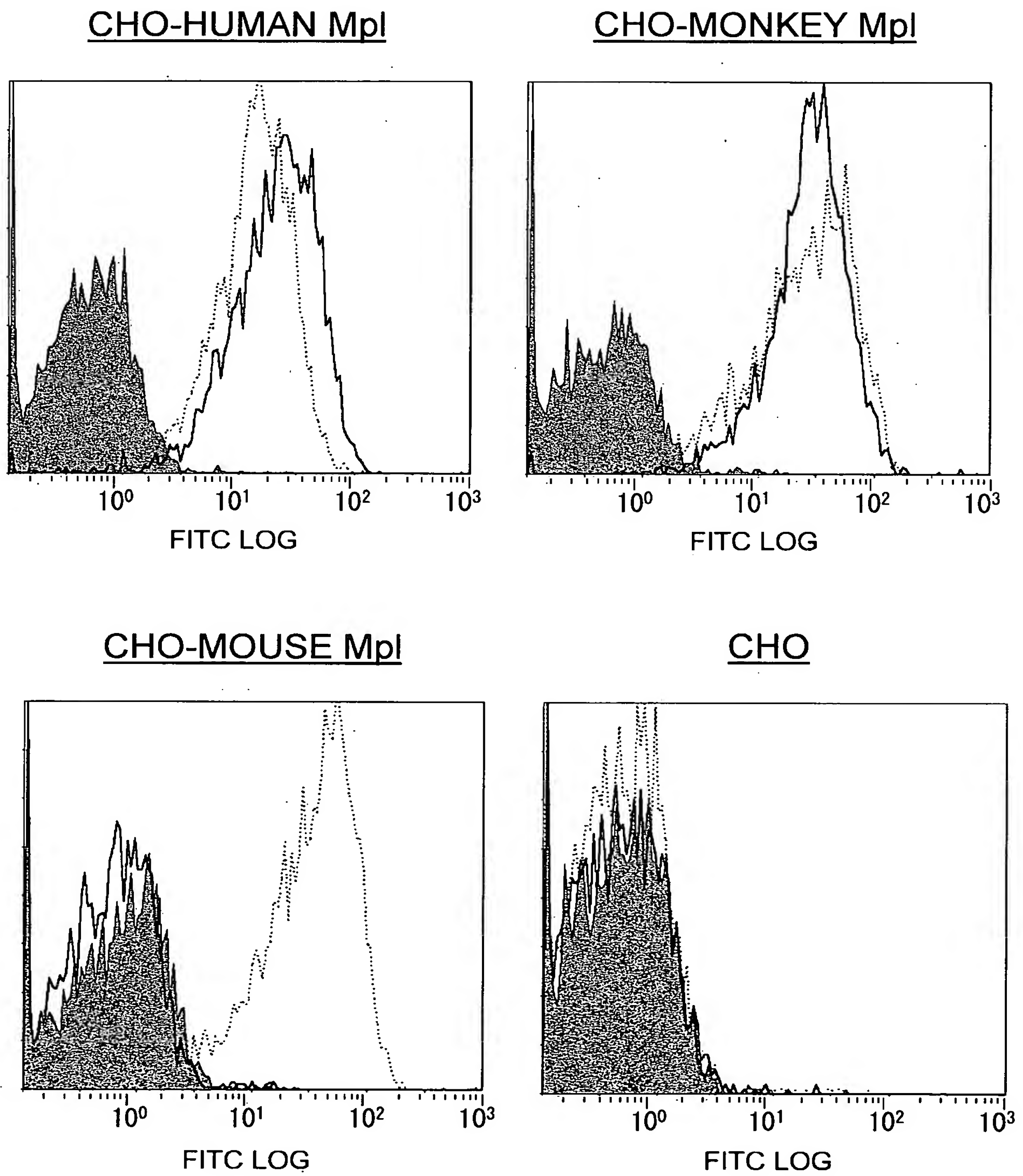
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VA130	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VA259	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB17B	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB12B	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB140	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB33	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB45B	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB8B	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB115	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB14B	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB22B	DIVMTQAAPSI	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB16	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB157	DIVMTQAAPSV	SVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB4B	DIVMTQAAPSV	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS
VB51	DIVMTQAAPSL	PVTPGESVSISC	RSSKSLH	SNGNTYLY	WFLQRP	QSPQLLIY	RMSNLAS

		CDR3				
VA7	GVPDRFSGSG	SGTAFTLRISRVEAEDVGIIYYC	MQHLEY	PFT	FGTG	TKLEIK
VA130	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VA259	GAPDRFSGSG	SGTAFTLRISRVEDVDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB17B	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB12B	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB140	GVPDRFSGSG	SGAAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB33	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB45B	GVPDRFSGSG	SGAAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB8B	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHVEY	PYT	FGSG	TKLEIK
VB115	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB14B	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
VB22B	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHIEY	PFT	FGSG	TKLEIK
VB16	GVPDRFSGSG	SGTAFTLTISSVEAEDVGVIYYC	MQHLEY	PYT	FGSG	TKLEIK
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VB4B	GVPDRFSGSG	SGTAFTLRISRVEAEDVGVIYYC	MQHIEY	PFT	FGSG	TKLEIK
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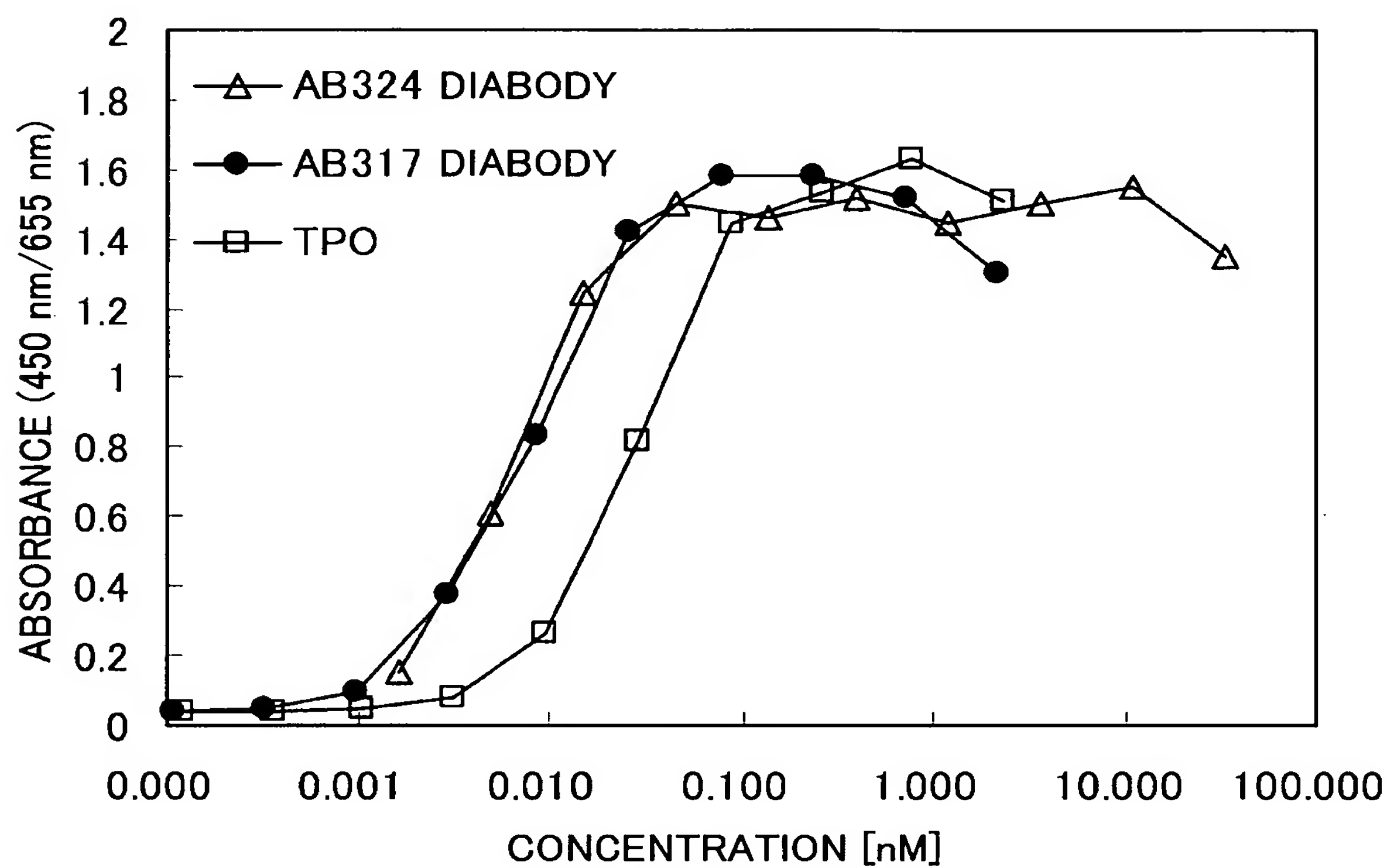
[Fig. 7]

		CDR1	CDR2
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VA130	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VA259	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB17B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB12B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB140	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB33	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB45B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB8B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB115	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB14B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB22B	DIVMTQAAPSIPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB16	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB157	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB4B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
VB51	DIVMTQAAPSLPVTPGESVSISC	RSSKSLHLSNGNTYLY	WFLQRPQGSPQLLIY
		CDR3	
VA7	GVPDRFSGSGSGTAFTLRISRVEAEDVGYIYYC	MQHLEYPFT	FGTGTKLEIK
VA130	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VA259	GAPDRFSGSGSGTAFTLRISRVEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB17B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB12B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB140	GVPDRFSGSGSGAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB33	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB45B	GVPDRFSGSGSGAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB8B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHVEYPYT	FGSGTKLEIK
VB115	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB14B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB22B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHIEYPFT	FGSGTKLEIK
VB16	GVPDRFSGSGSGTAFTLTISSVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB157	GVPDRFSGSGSGTAFTLKISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB4B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHIEYPFT	FGSGTKLEIK
VB51	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK

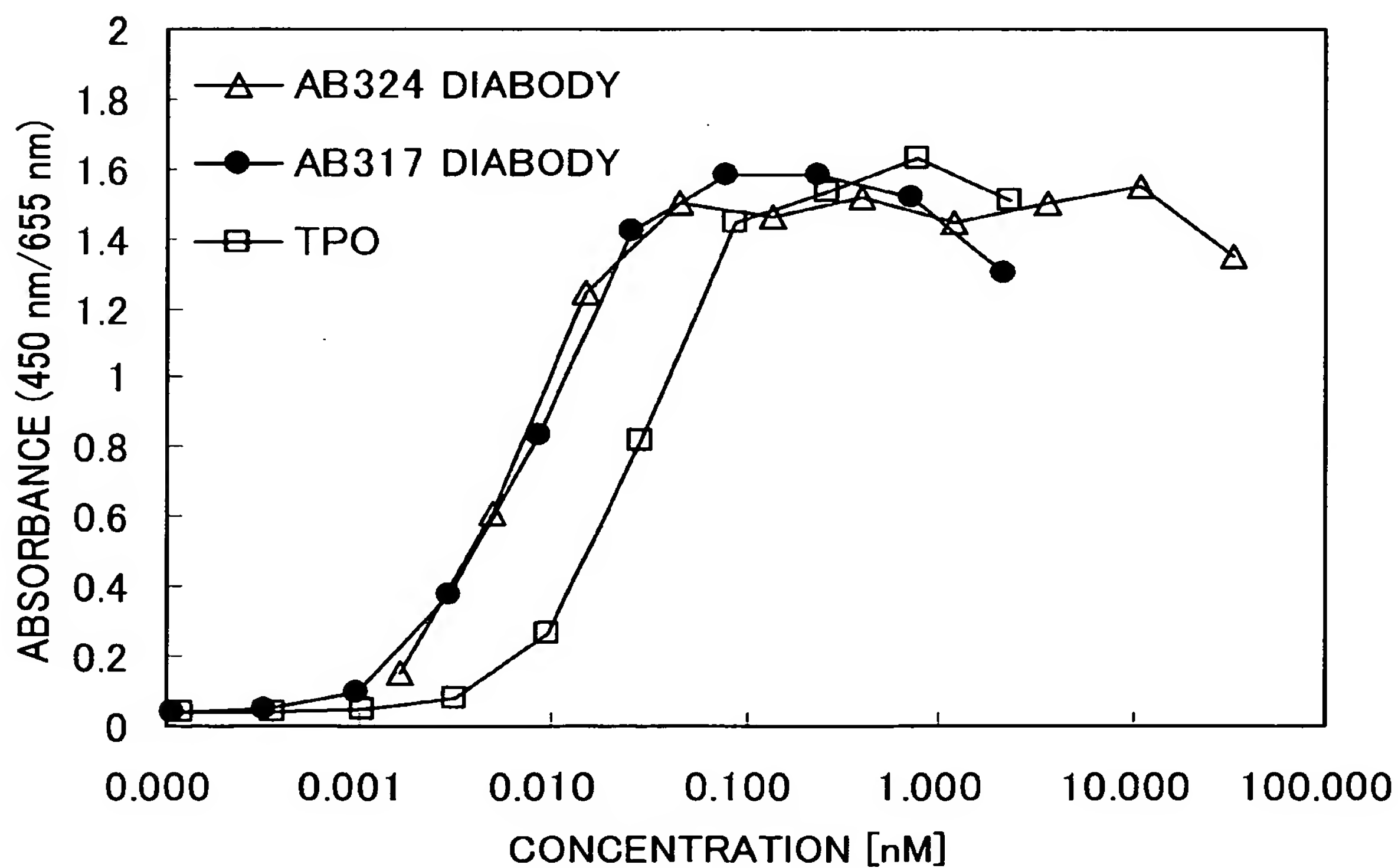
[Fig. 8]



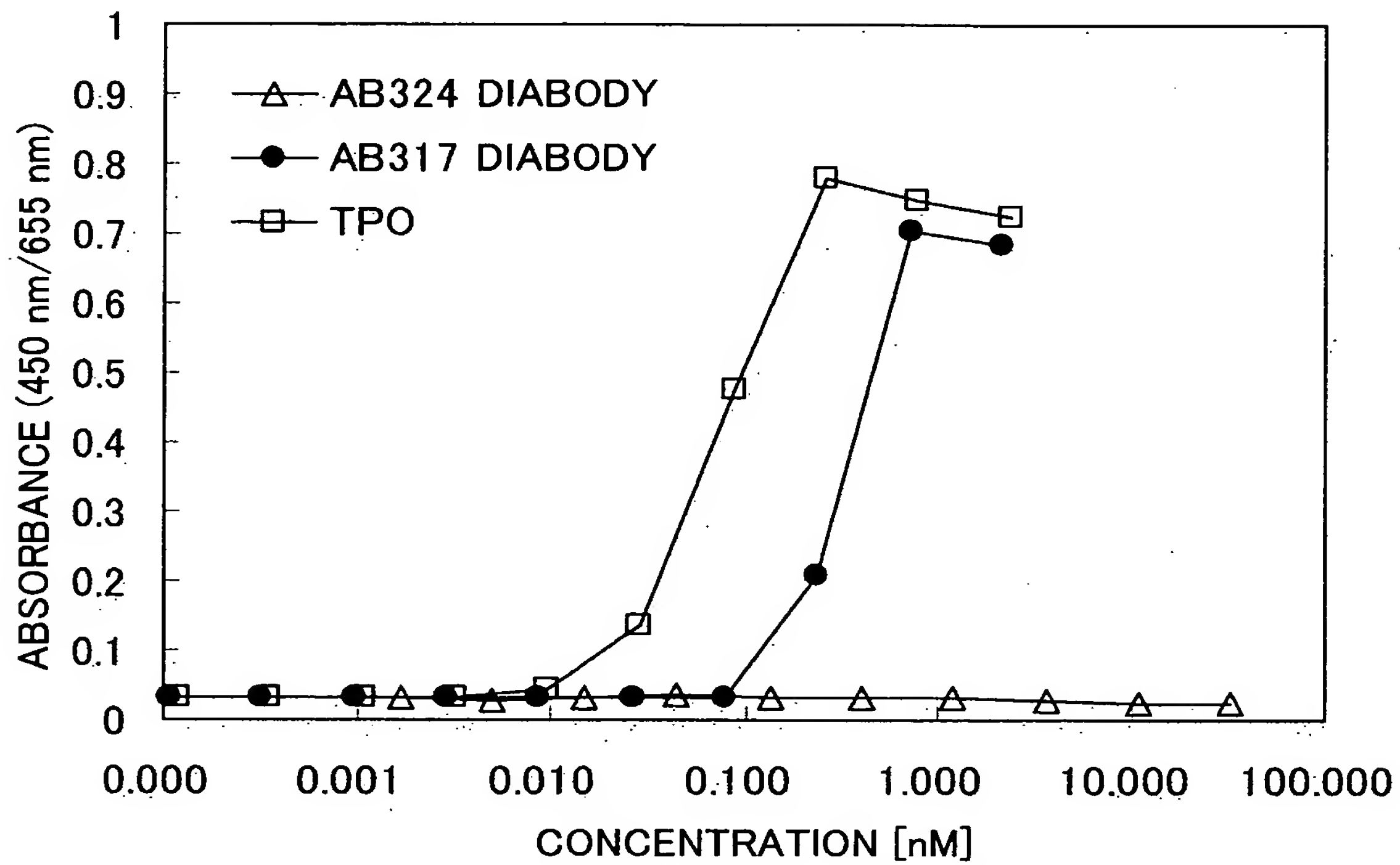
[Fig. 9]



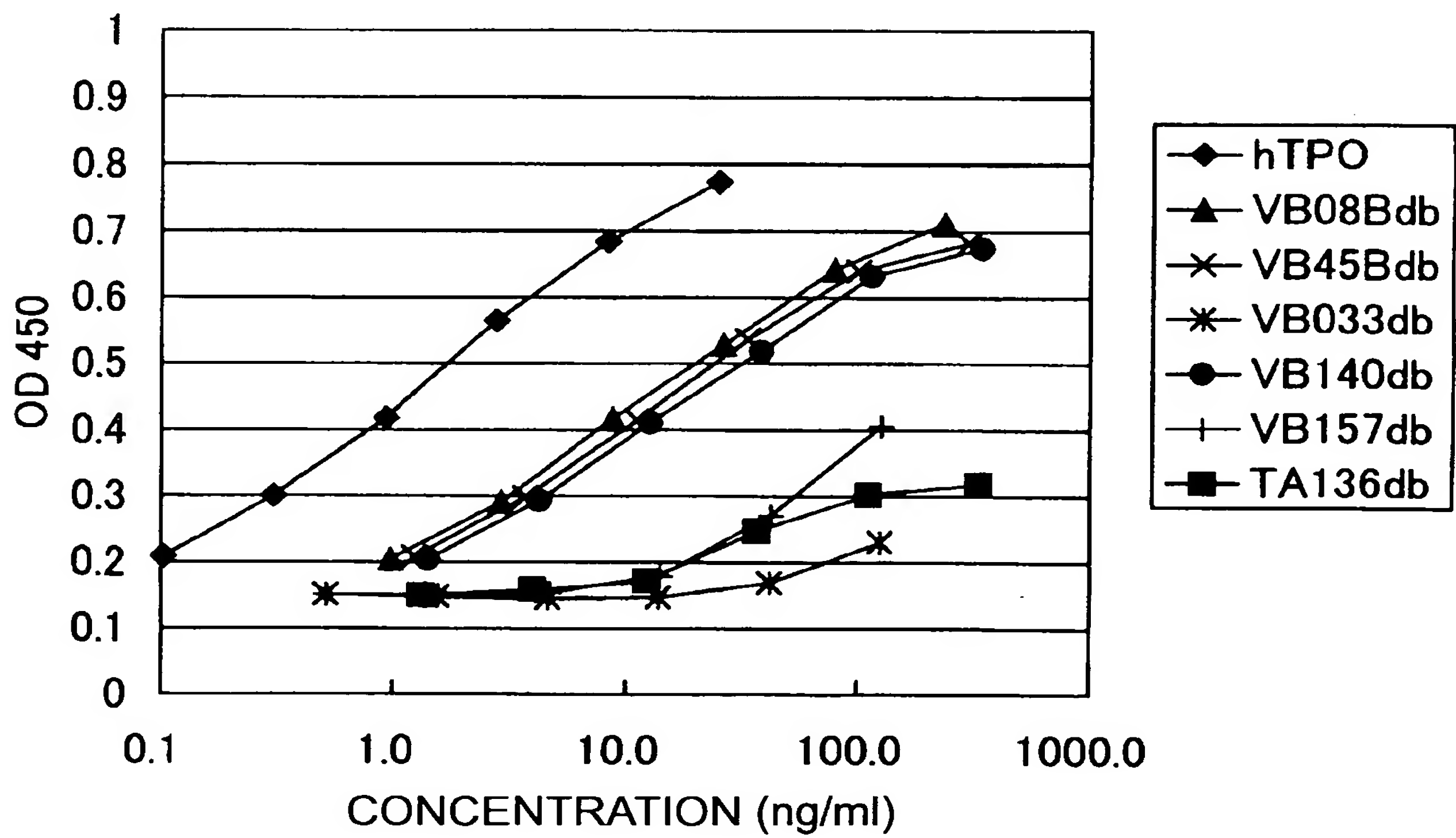
[Fig. 10]



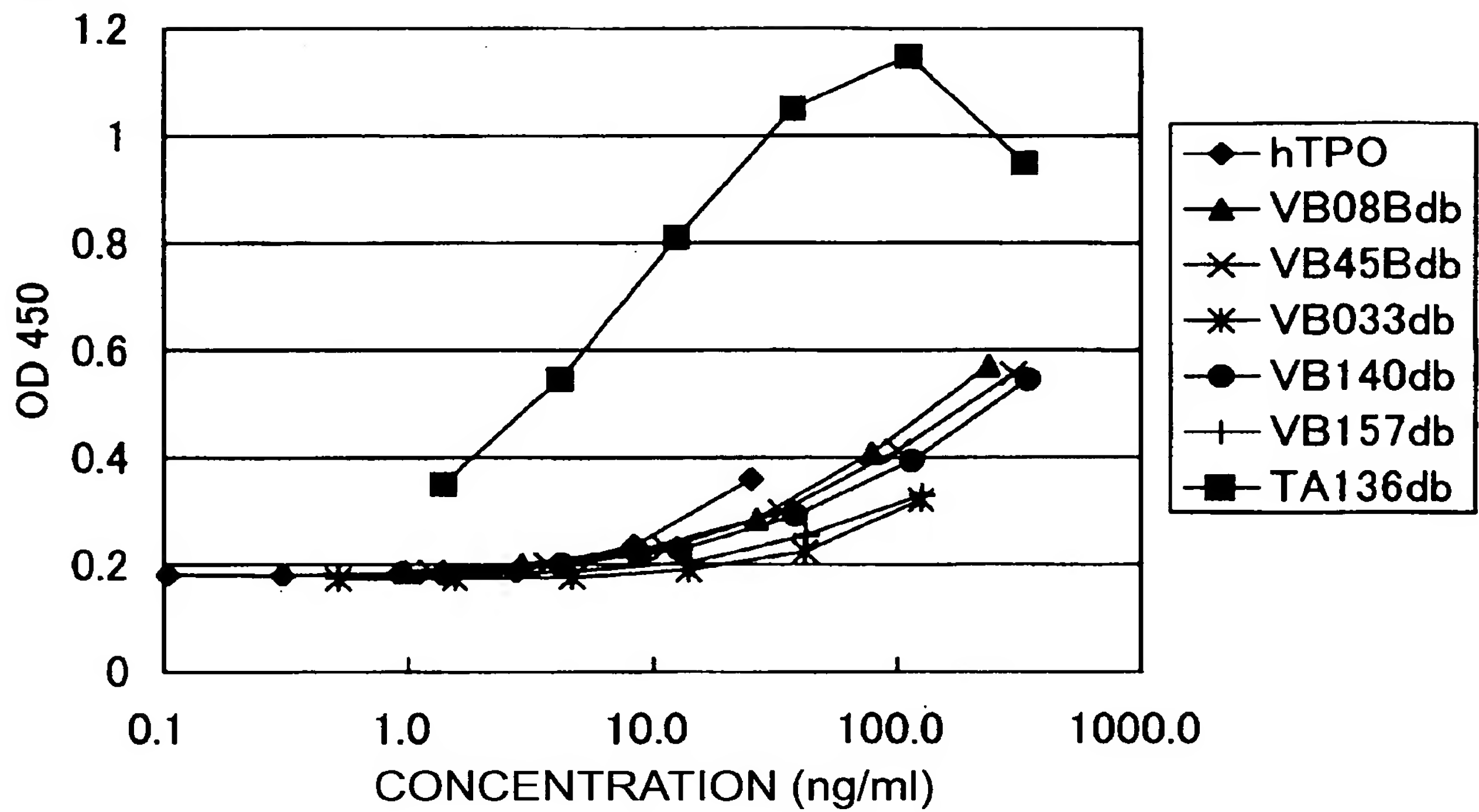
[Fig. 11]



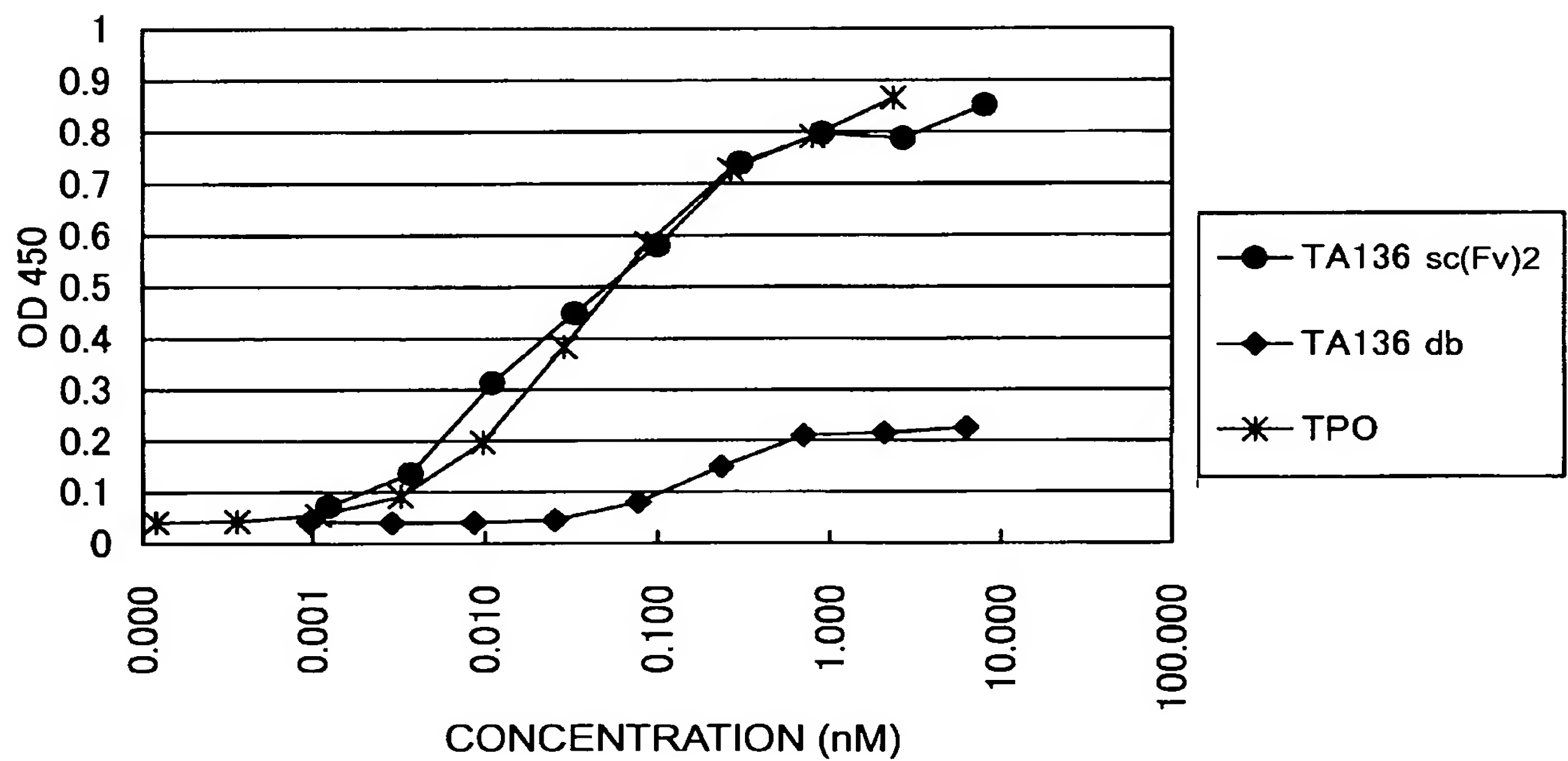
[Fig. 12]



[Fig. 13]

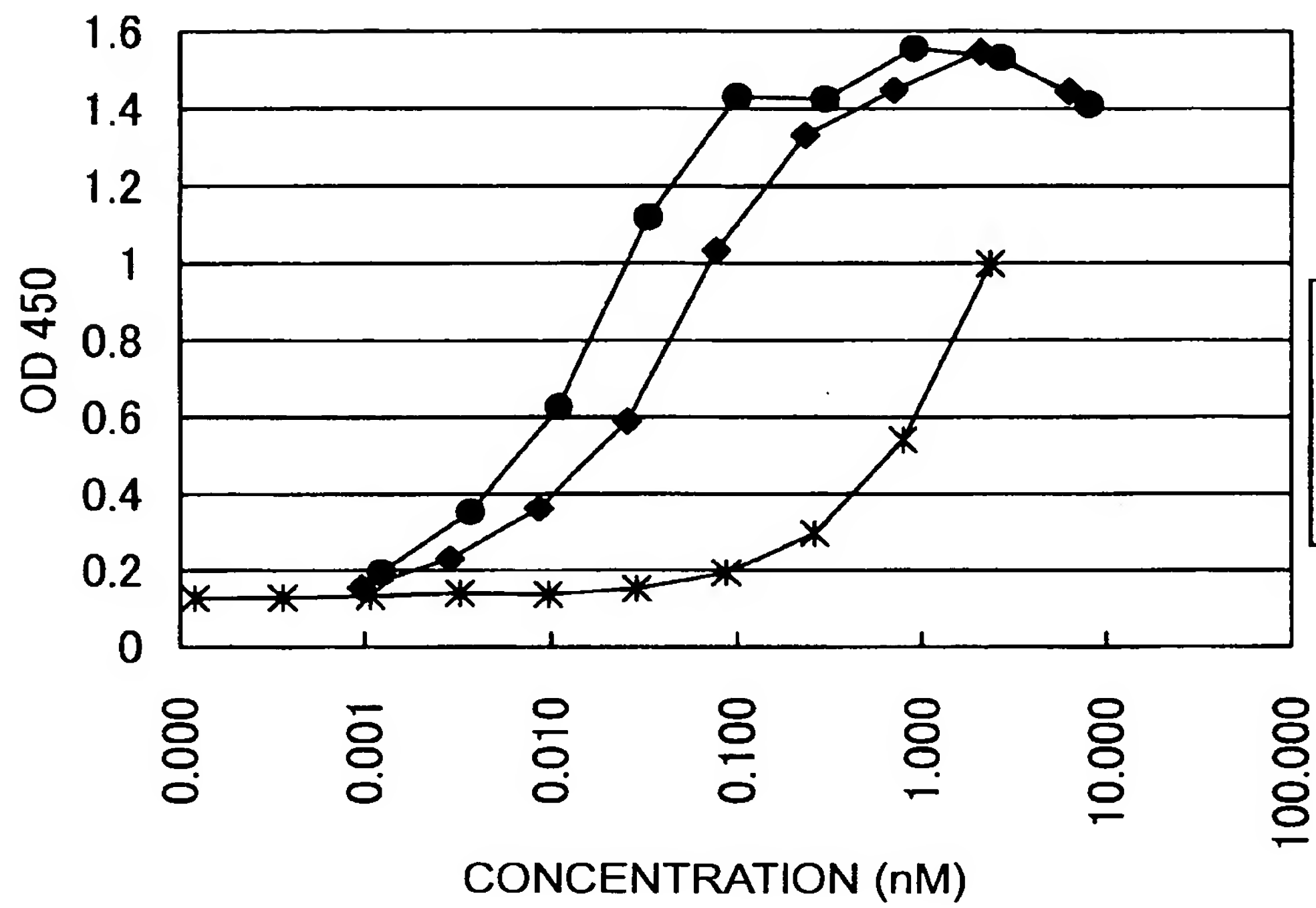


[Fig. 14]

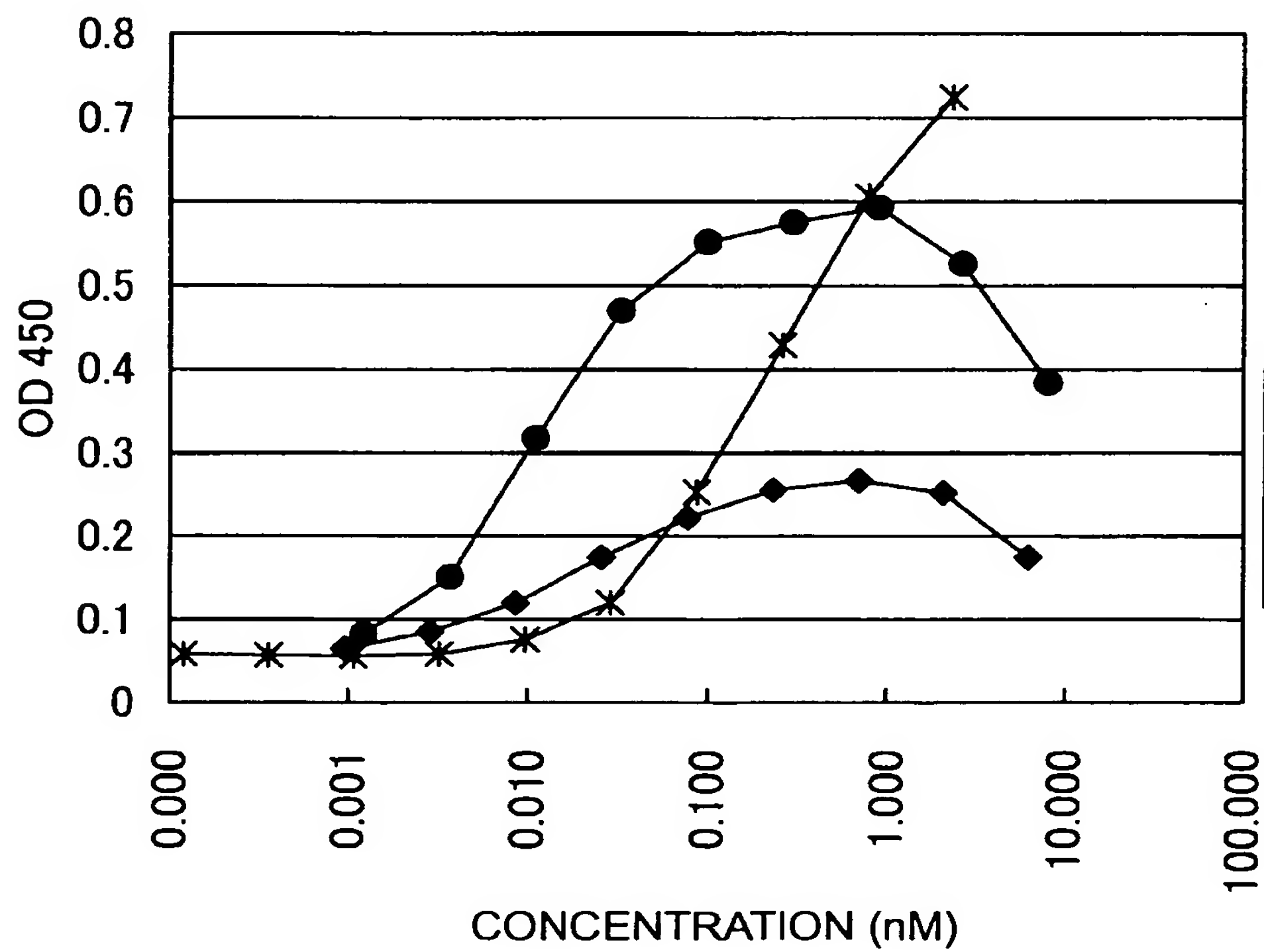




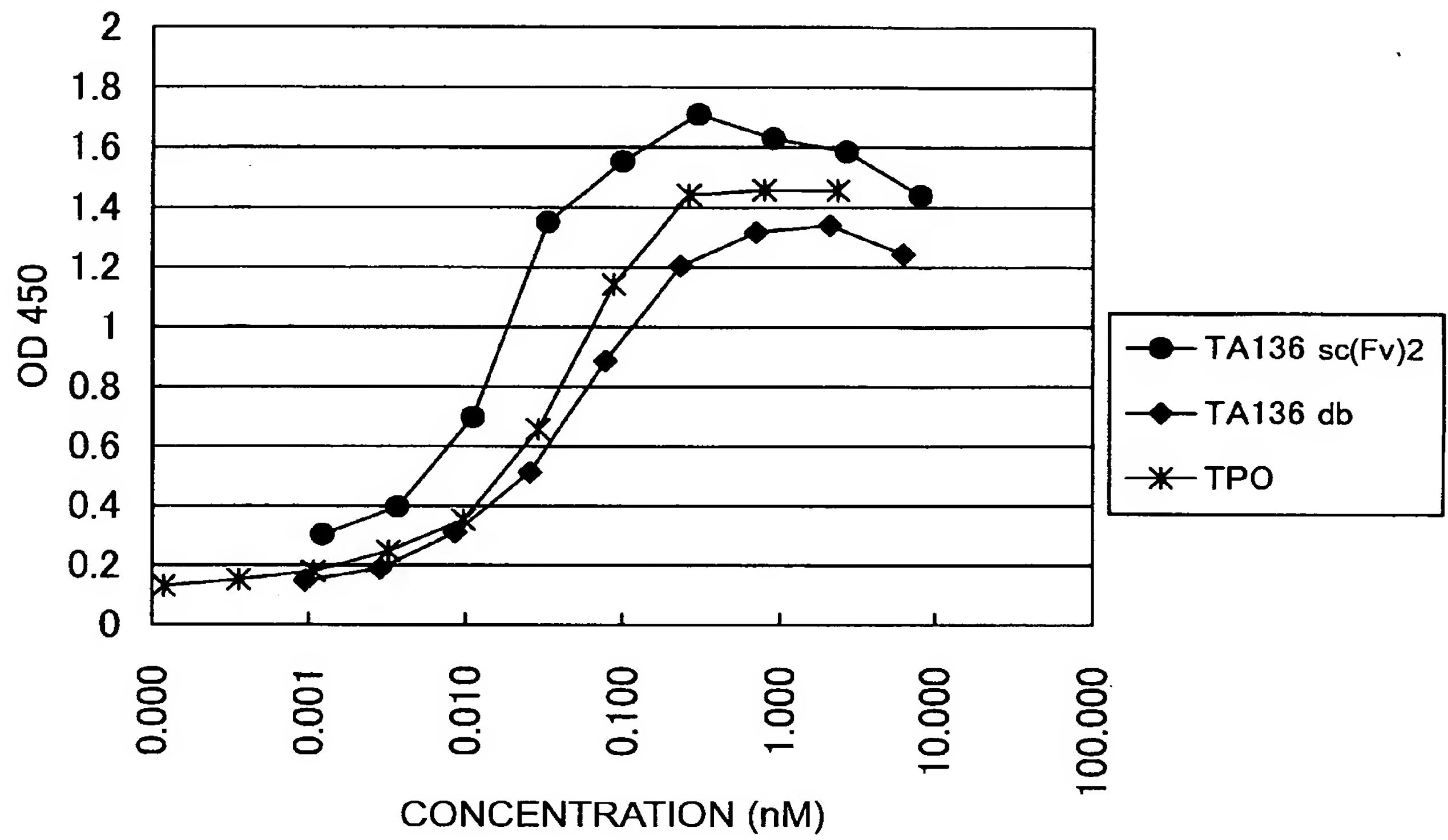
[Fig. 15]



[Fig. 16]



[Fig. 17]

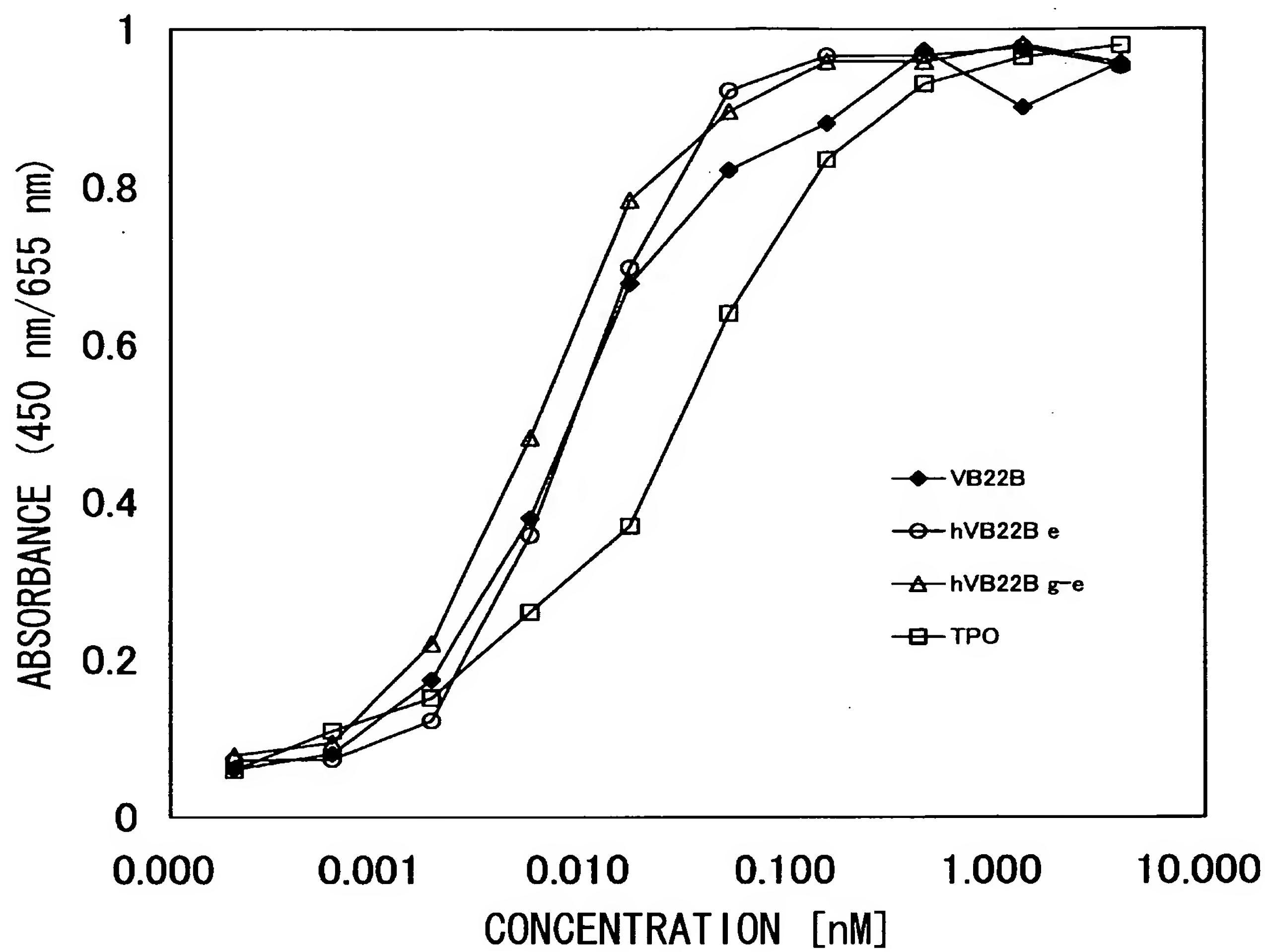


[Fig. 18]

HUMANIZED HEAVY CHAIN  
QVQLVQSGPEVKKPGASVKVSCKASGYTFTNSWMNHWVRQRPCKGLEWMGRYPGDGETIYNGKFRVVRVTITADESTAYMELSSLRSEDTAVYYCAR[CYDDYSFAYWGQGT]TVTVSS  
FR1 CDR1 FR2 CDR2 FR3 CDR3 FR4

HUMANIZED LIGHT CHAIN  
DIVMTQSALSPLPTGEPASISCRSSKSLHSHNGNTYLYWFFQKPGQSPQLLIYRMSNLA[SGV]PDRFSGSGSGTAFTLKISRVEAEDVGVYYC[MQHIEY]PFTTFGQGTKLEIK  
FR1 CDR1 FR2 CDR2 FR3 CDR3 FR4

[Fig. 19]



[Document Name] Abstract

[Abstract]

5 [Problems to be Solved] An objective of the present invention is to provide novel anti-Mpl antibodies having TPO-agonistic activity.

[Means for Solving the Problems] Anti-human Mpl antibodies were isolated and purified, and then anti-human Mpl diabodies and anti-human Mpl sv(Fv)<sub>2</sub> were purified using genetic engineering techniques. Furthermore, the present inventors succeeded in humanizing anti-human Mpl sc(Fv)<sub>2</sub>.

10 The diabodies and sc(Fv)<sub>2</sub> were assayed for TPO-like agonistic activity, and were found to have activities higher than those of anti-human Mpl antibodies, or activities equivalent to or higher than those of naturally-occurring human TPO ligand.

[Selected Drawings] None